The influence of social and environmental factors on the stress response and development in juvenile & larval lake sturgeon, *Acipenser fulvescens*

by

Ahmed Waheed

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Department of Biological Sciences
University of Manitoba
Winnipeg

Abstract

This thesis has examined the influence of the environment on aspects of the acute stress response in juvenile and pro-larval *Acipenser fulvescens*.The acute stress response was examined in grouped and isolated juveniles. Catecholamines significantly increased in both treatments one minute post-stress and plasma glucose was significantly higher in isolated as compared to grouped fish one minute post-stress. In the second series of experiments fertilized eggs of *A. fulvescens* were raised at 9, 12 & 15°C. Chromaffin-like cells were studied using light and electron microscopy techniques. Development of renal tissue was also examined in these treatment groups. Two populations of chromaffin-like cells were identified, one in close association with the proximal tubule of the kidney, and the other in close association with the neural tube. Results suggest this latter population were immature pheochromoblast like-cells. Development of renal tissue followed a predictable pattern that was most rapid in the 15°C treatment.

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Objectives

The objectives of the current thesis were to further explore the role of catecholamines in the lake sturgeon, *Acipenser fulvescens*. Specifically the effect of stress on catecholamine release was examined in juvenile lake sturgeon. I have tested the hypothesis that social environment will influence the catecholamine stress response in juvenile lake sturgeon. Furthermore, identification of chromaffin and kidney tissue in developing lake sturgeon larvae was conducted and I attempted to test the hypothesis that environmental factors such as temperature may influence the development of chromaffin and renal tissue.

Chapter 1: General Introduction

1.1 – ***Stress in fishes***

The word stress can be defined in many ways. Biologically it refers to any event that causes a series of hormonal changes in an organism in order to regulate key physiological processes in response to a long or short term stressor (Pickering, 1981). In the aquatic environment fish go through various long and short term stresses that can be either biotic (e.g.: predation or parasitism) or abiotic (e.g.: changes in dissolved oxygen or temperature) in nature. These stressors can also be acute or chronic and each will result in vast differences in the physiological impact on the fish. Therefore, depending upon the duration and severity of individual stressors, fish go through various physiological and hormonal changes in response to the stressor that will alter homeostatic mechanisms and influence the organism from the level of the cell to the level of the population (Iwama *et al.*, 2006).

This change is broadly divided into a three phased response: primary, secondary and tertiary (Iwama *et al.*, 2006, Mazeaud *et al.*, 1977). The primary response, is the first and starts with the perception of a stressful event and coincides with an initial increase in circulating levels of stress hormones (Reid *et al.*, 1998). Initiation of the primary stress response was first identified by Walter Cannon in the early 1900's (Cannon, 1929) and is perhaps best known as the "fight or flight" response and is considered a good adaptation. Much research has been done to identify these stress hormones and they have been divided into two major groups; catecholamines and glucocorticoids. In addition, thyroxine, prolactin and somatolactin have been implicated in the primary stress response in fish (Barton, 2002), however, this thesis will focus on the role of the glucocorticoids and in particular the catecholamines in the stress response in fish.

The primary response is succeeded by the secondary response, again considered a positive adaptation by the organism, and this involves the action of glucocorticoids and catecholamines at the target sites, influencing various biochemical pathways and transcriptional processes. Glucocorticoids are known to act through nuclear receptors by

way of heat shock proteins acting as intracellular chaperone proteins (Barton, 2002, Parsell and Lindquist, 1993); whereas catecholamines exert their actions through G-protein linked adrenoceptors on the cell membrane (Reid *et al.*, 1998). The net result is that the hormones prepare the fish to survive in the face of change, i.e., the stressor. These primary and secondary responses are summarized in figure 1.1, which will be discussed in further detail in succeeding sections. If the stressor persists it reaches the tertiary stage impacting the immune response, behavior, reproductive ability and ultimately growth of the population. If the tertiary stage continues it is considered a chronic stress and maladaptive to the fish (Iwama *et al.*, 2006).

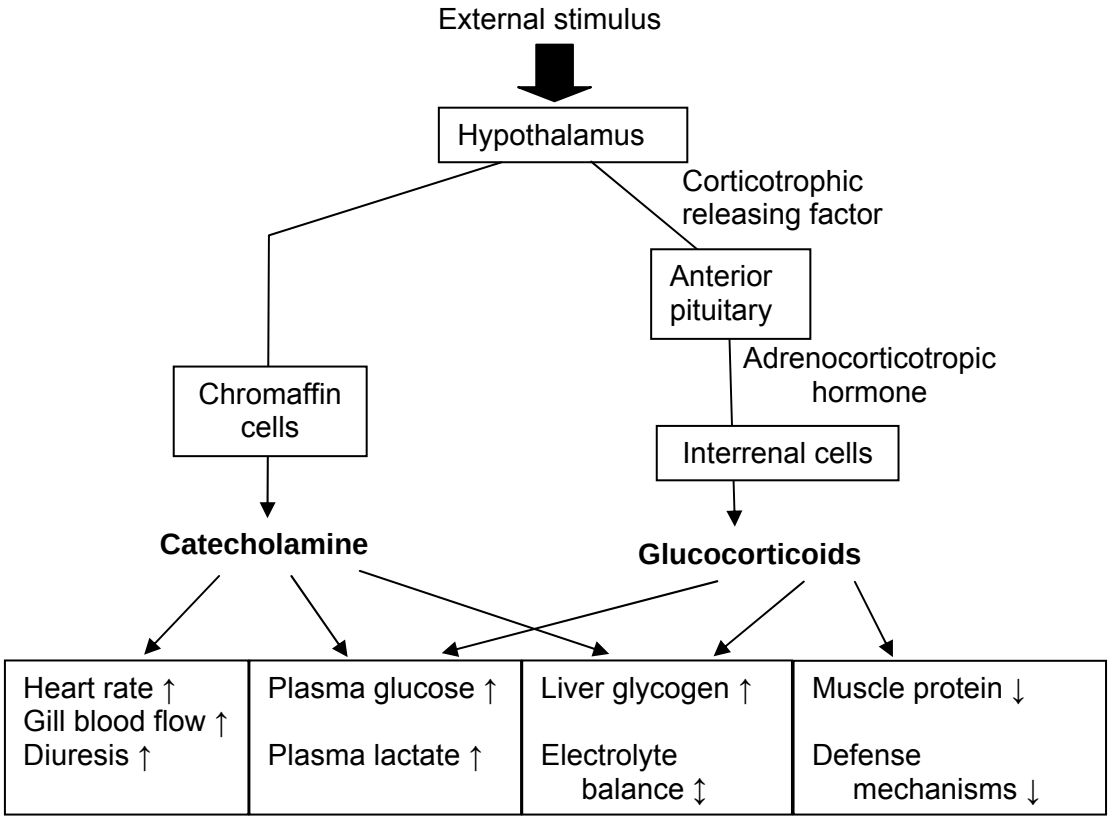


Figure 1.1: Summary of the primary and secondary effects of stress in fish. (↑), Increase; (↓), decrease. Figure adapted from Mazeaud et al. (1977).

1.1a Catecholamines:

The catecholamines are a group of hormones that are rapidly secreted in response to various stressors such as, exercise, predator-prey interaction, temperature change, etc. The major bioactive components are epinephrine (EPI) (or adrenaline), norepinephrine

(NE) (or noradernaline) and dopamine (Dp) that are secreted in different concentrations and often result in distinctly different physiological endpoints. In contrast to other stress hormones they are secreted rapidly into the circulation within seconds following exposure to a stressful event. They have a short half life of minutes and their metabolites are secreted primarily in urine either in the free form or conjugated as sulphates or glucuronates (Mazeaud and Mazeaud, 1981, Nilsson, 1984).The initial discovery of catecholamines occurred in 1895 when EPI was identified by a Polish physiologist Napoleon Cybulski (Skalski and Kuch, 2006). Two years later a North American scientist, John Jacob Abel isolated it from human plasma. However, Dp was not discovered until close to 50 years later (Carlsson *et al.*, 1958). Interestingly, Carlsson and his coworkers thought that Dp was the precursor of NE, and played no physiological role. However, it has since been well described as a bioactive hormone independent of the actions of both EPI and NE particularly in the brain (Benes, 2001). The name catecholamine is derived from the catechol and amide moieties (Flatmark, 2000). The catechol portion consists of a 3,4-dihydroxyphenyl nucleus with an ethyl chain attached to it at carbon number 1. An amino group is present at the end of each carbon chain, grouping these hormones as biogenic amines. Histamine and serotonin are also considered as biogenic amines. Structurally they differ from each other by the nature of the ethyl chain attached to the catechol group. Dp lacks the hydroxyl group on its β-carbon, while EPI contains an extra methyl group attached to the amide group (Figure 1.2).

Synthesis of catecholamines involves a four step reaction pathway, starting with the amino acid tyrosine and finishing with EPI. The whole pathway occurs in a highly specialized group of cells originating from neural crest cells in fishes (Reid *et al.*, 1998) that impart a yellow to brown color following staining with chromate reagents (Hillarp and Hokfelt, 1955, Lever *et al.*, 1976, Wood *et al.*, 1971), hence why the catecholamine secreting tissue is also known as chromaffin tissue. These cells are basophilic in nature and thus stain darkly with stains such as haematoxylin (Clark *et al.*, 2005, Coupland *et al.*, 1976, Kobayashi and Coupland, 1993, Tang *et al.*, 2009). Blaschko first recognized chromaffin tissue in the adrenal medulla of mammals (Blaschko, 1939). However, in fishes there is no discrete organ for their location.

In teleost fishes the tissue is dispersed within the cephalic portion of the kidney also known as the head kidney (Wendelaar-Bonga, 1997). However, in more primitive fishes, the tissue is associated within major blood vessels such as the posterior cardinal veins (PCV) (Euler and Fange, 1961, Perry *et al.*, 1993).

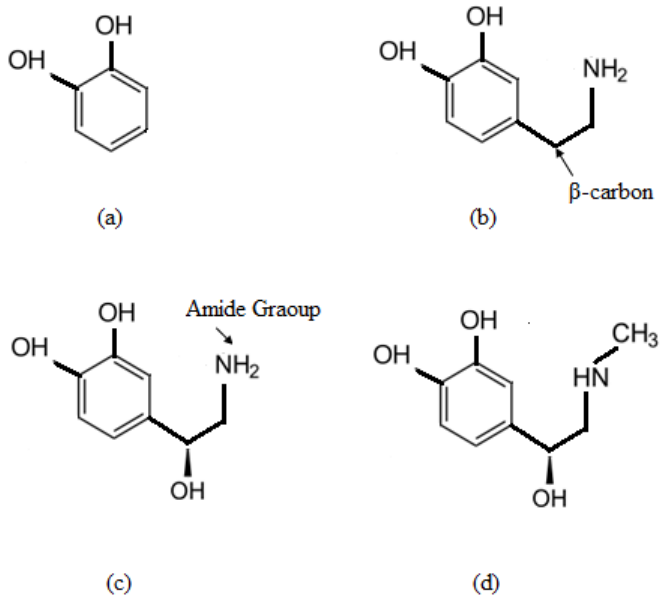


Figure 1.2: Structural representation of the catechol precursor molecule (a) for, Dopamine (b), Norepinephrine (c) and epinephrine (d). Source: http://en.wikipedia.org/wiki/Catecholamine. Figure adapted from, Nagatsu (1973b)

The catecholamine synthesis pathway (sometimes referred to as the Blaschko pathway), explained in figure 1.3, begins with tyrosine which then undergoes a hydroxylation reaction and is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH) taking tetrahydrobiopterin as a cofactor and converting it to dihydrobiopterin (Abou-Donia *et al.*, 1982, Nagatsu *et al.*, 1964). TH is the principle rate limiting enzyme of the entire pathway and is highly sensitive to various positive and negative feedback signals (Kvetnansky *et al.*, 2009). Decarboxylation on the side chain then converts DOPA to Dp by the enzyme DOPA decarboxylase, indeed this was the first enzyme to be discovered in the catecholamine biosynthetic pathway (Blaschko, 1939). In EPI and NE secreting cells, Dp is further converted into NE by hydroxylation at the β-carbon of the side chain. The enzyme needed in this reaction is dopamine β-hydroxylase (DBH), and was first recognized by Kaufman and Friedman (1965). NE is then converted

into EPI by methylation at the amino group present at the end of the ethyl chain by the help of the enzyme phenylethanolamine N-methyltransferase (PNMT) serving S-adenosylmethionine as a methyl donor (Wong *et al.*, 1987).

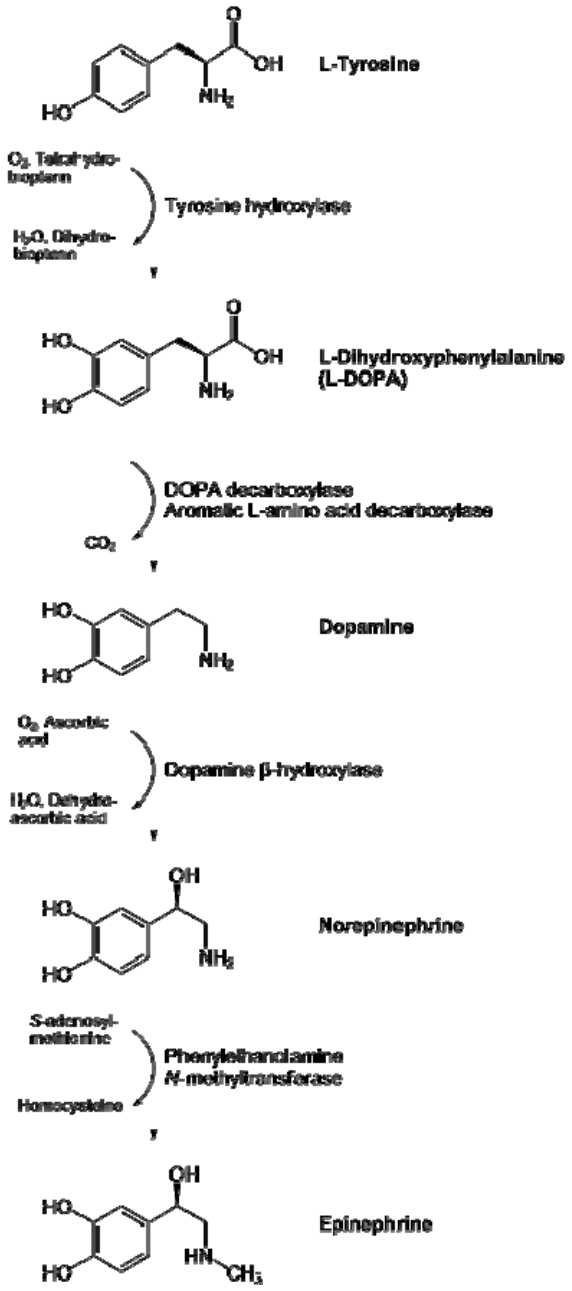


Figure 1.3: Blaschko pathway. Source: http://en.wikipedia.org/wiki/Catecholamine. Figure adapted from Nagatsu (1973a)

All the described enzymes in the EPI synthesis pathway have been identified in teleost fishes, particularly rainbow trout, *Oncorhynchus mykiss*, and show similar characteristics to mammals (Nilsson, 1984), however, the optimum temperature necessary for enzyme activity differs from mammals (Jonsson, 1983). Moreover, in Atlantic cod, *Gadus morhua*, the rate limiting enzyme identified in this pathway is PNMT rather than TH (Jonsson and Nilsson, 1983a, b).

Catecholamines perform a variety of functions in the body through adrenoceptors. The two major subgroups of these receptors are the alpha (α) and beta (β) adrenoceptors. In fish as in higher animals, adrenoceptors are G-protein linked receptors influencing a variety of intracellular effector proteins depending of the nature of the receptor that is stimulated (Owen *et al.*, 2007, Siebenaller, 2003, Srirangalingam and Chew, 2008). α-adrenoceptors are further subdivided into α_1 and α_2-adrenoceptors, while β_1, β_2 and β_3-adrenoceptors are identified as the β-adrenoceptor subtypes. These receptors have been identified in mammals, reptiles, amphibians and birds, with our greatest understanding being obtained from mammalian studies. Therefore, our understanding of the role of α and β adrenoceptors in fish lags far behind that in mammals.

The vast majority of research regarding catecholamines in fish has focused on rainbow trout, and known physiological effects of the activation of the adrenergic system include: red cell swelling and enhancement of blood oxygen carrying capacity (Owen *et al.*, 2007, Perry and Reid, 1992); reduction of plasma pH and increase in partial pressure of oxygen via a decrease in hemoglobin-oxygen binding capacity of blood (Primmett *et al.*, 1986, Tang *et al.*, 1989); increase in cardiac output via positive ionotropic (force of contraction) and chronotropic (heart rate) effects on the heart (Farrell *et al.*, 1982, Farrell *et al.*, 1986); hyperglycemia by activating hepatic glycogenolysis (Fabbri *et al.*, 1998, Perry *et al.*, 1988, Wright *et al.*, 1989); lipolysis with an increase in free fatty acids and glycerol (Fain and Garciasainz, 1983); and vasoconstriction in the peripheral circulation and dilation of gill vasculature (Sandblom *et al.*, 2010, Zhang *et al.*, 1998). Almost all of these actions prepare the fish to rapidly cope with an environmental stressor in a fight or flight response. Interestingly, much of the same research conducted in teleost fish produces the opposite effect or has no effect at all in more ancient fishes such as the

agnathans and elasmobranchs (Perry and Bernier, 1999). Details of these effects in these species will be discussed in the following sections.

1.1b Corticosteroids:

Corticosteroids are the second major group of hormones secreted in response to a stressful event. Compared to the catecholamines they have a longer lag time making them more suitable for measuring the stress response in fish (Gamperl *et al.*, 1994). In mammals these hormones are secreted from the adrenal cortex situated at the poles of the bilateral kidneys. They are divided into mineralocorticoids and glucocorticoids consisting of aldosterone and cortisol respectively as the major circulating hormones. Fish lack aldosterone (Bern, 1967, Sandor *et al.*, 1966) and therefore corticosteroids perform a dual function regulating both mineral and energy balance (Wendelaar-Bonga, 1997). Different forms of corticosteroid hormones have been identified in fish, including cortisol, cortisone, corticosterone, 11-deoxycortisol, 20β-dihydrocortisone, alloTHE (5α-pregnane-3α,17α,21- triol-11,20-dione), allo-α-cortolone (3α,17α,20α,21-tetrahydro-5α-pregnan-11-one), and allo-β-cortolone (3α,17α,20β,21-tetrahydro-5apregnan- 11-one) (Gamperl *et al.*, 1994, Mazeaud and Mazeaud, 1981, Webb *et al.*, 2007). In teleost and chondrostean fishes, cortisol is the principle circulating corticosteroid (Barton, 2002, Barton *et al.*, 1998, Patino *et al.*, 1987) while the unique 1α-hydroxycorticosterone acts as the principle circulating corticosteroid in elasmobranch fish (Idler and Truscott, 1966, 1967, Truscott and Idler, 1972). All these steroids share a common pathway for secretion, that is, from the interrenal gland. The interrenal gland in fishes is the homologue to the mammalian adrenal cortex and is concentrated in the head kidney of teleost fish (Abdel-Aziz *et al.*, 2010a, b), and located throughout the whole kidney of chondrosteans and elasmobranchs as discrete globular units (Barannikova *et al.*, 1978, Idler and Ohallora.Mj, 1970, Youson *et al.*, 1976). As in higher vertebrates the initial trigger for stimulation of the Hypothalamic-pituitary-interrenal (HPI) axis is the release of corticotropin releasing hormone (CRH) sometimes referred to as corticotrophic releasing factor (CRF) from hypothalamic CRH neurons. CRH then acts on the pituitary gland to stimulate the secretion of adrenocorticotropic hormone (ACTH), which in turn acts upon the interrenal tissues to stimulate the secretion of cortisol (Figure 1.1) (Norris and Hobbs,

2006, Schreck *et al.*, 1989). Cortisol is then conjugated or metabolized and excreted by renal or intestinal routes (Patino *et al.*, 1987). There are various positive and negative feedback regulatory loops which operate at different levels to control the secretion of glucocorticoids including among others, catecholamines and serotonin (Figure 1.4) (Carruth *et al.*, 2002, Guerriero and Ciarcia, 2006). In addition to ACTH, α-melanocyte stimulating hormone, growth hormone, thyroxine, angiotensin II, arginine vasotocin, catecholamines, atrial natriuretic peptide, and urotensin I have all been shown to influence the synthesis and release of cortisol in teleost fish (Balm *et al.*, 1994). Furthermore, factors produced by the immune system may also modulate cortisol secretion either directly or indirectly by affecting different components of the HPI axis (Hazon and Balment, 1998).

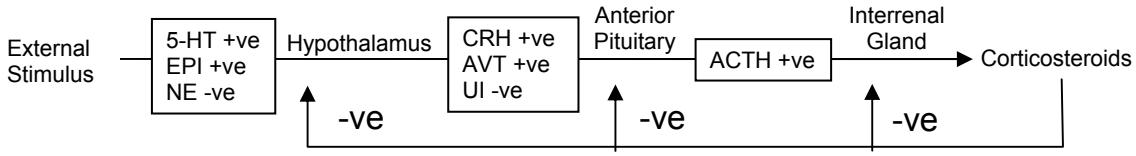


Figure 1.4: HPA axis of fish. Solid lines show positive feedback and dashed lines show sites of negative feedback. +ve, stimulatory; −ve, inhibitory; 5-HT, serotonin; ACTH, adrenocorticotropic hormone; AVT, arginine vasotocin; CRH, corticotropin releasing hormone; EPI, epinephrine; NE, norepinephrine; UI, urotensin I. Figure adapted from Carruth et al. (2002).

Corticosteroids exert their action at target tissues via interaction with nuclear corticosteroid receptors (CR). Depending on the nature of action these receptors are divided into mineralocorticoid receptors (MR) and glucoocorticoid receptors (GR). Various molecular studies in teleost fish have shown two isoforms of GR, namely GR I and GR II (Bury *et al.*, 2003, Greenwood *et al.*, 2003). CR's are heteromeric in nature and are largely retained in the cytoplasm in an unbound and inactive form where they have a high affinity for cortisol. In this state heat shock proteins are bound to the receptor and are involved in preserving the structural integrity of the receptor in the cytosol of the cell. MR's and GR's share similar structural components and functional domains. The carboxyl end of receptor contains the E domain that is responsible for hormone attachment and hormone dependent trans-activation while at amino-end, the A/B domain modulates transcriptional activity. The C domain, present at the central DNA binding

region, is responsible for DNA binding and receptor dimerization and the D domain is involved in conformational changes of the receptor (Bury and Sturm, 2007, Prunet *et al.*, 2006). CR preferentially binds cortisol and induces a glucocorticoid response. MR induces a response closer to the action of mineralocorticoids. Following cortisol binding the receptor undergoes a conformational change, leading to the dissociation of the receptor ligand complex from the chaperone proteins, and translocation into the nuclear space. On entering the nucleus, the ligand-receptor complex binds to specific sites on the DNA known as 'hormone-response elements', and as a result can influence the expression of a number of genes. GR's can be homodimers in nature and consist of highly conserved modular domains that perform specific functions, thus can cause either transcriptional activation or repression of target genes depending on the specific required response. In hepatocytes, cortisol has the direct action of augmenting expression of rate limiting enzymes for gluconeogenisis by translation while on the other hand they also inhibit the formation of rate limiting enzymes for glycogenesis by gene suppression, producing an overall state of hyperglycemia (Donaldson, 1981).

1.2 - ***Chromaffin tissue location and catecholamine release in ancient fishes***

1.2a Chromaffin tissue in ancient fishes:

In mammals chromaffin tissue is found in a specific organ, the adrenal medulla (Ba-Omar and Downie, 2006, Clark *et al.*, 2005, Coupland, 1965, Tang *et al.*, 2009) and is highly innervated by the sympathetic nervous system (Llewellyn-Smith, 2009, Tomlinson and Coupland, 1990). Both light and electron microscopic studies of chromaffin tissue clearly demonstrate two different cell types containing NE and EPI (Wetzstein, 1957, Yates *et al.*, 1962). Under the light microscope, NE stains darker than EPI containing cells, when fixed with dichromate or iodate containing reagents (Gerard *et al.*, 1930, Lever *et al.*, 1976). Under the electron microscope NE containing cells show more electron dense spherical granules than EPI containing cells which have spherical to oval shaped granules. Both of these granule types are evenly distributed throughout the cytoplasm except the perinuclear area (Coupland, 1965, Coupland *et al.*, 1964). A third

set of cells has also been demonstrated but they are thought to contain various byproducts of the Blaschko pathway (Reid *et al.*, 1998).

Table 1.1 describes the concentrations of NE and EPI in $ng.mg^{-1}$ of chromaffin tissue in various fish species. In teleost fish chromaffin tissue is concentrated in small islet cells in the head kidney region in close association with the PCV (Gallo *et al.*, 1993, Nandi, 1961, Wendelaar-Bonga, 1997). Electron microscopy studies reveal the presence of both NE and EPI cells which are highly innervated by nerves where acetylcholine serves as a neurotransmitter (Crivellato *et al.*, 2006, Mastrolia *et al.*, 1984). EPI was found to be the major hormone concentrated in the area associated with the PCV than the other regions of the head kidney (Abrahamsson and Nilsson, 1976, Hathaway and Epple, 1989, Nakano and Tomlinson, 1967, Reid and Perry, 1994).

In elasmobranch fish chromaffin tissue was not identified until the 1960's when the activity of key enzymes involved in the biosynthesis, PNMT, and catabolism, catechol-O-methyltransferase of catecholamines (Mazeaud and Mazeaud, 1981, Wong, 2006) were measured in the interrenal bodies found throughout the kidney (Brown and Trams, 1968). Since then chromaffin tissue has also been identified in the sympathetic chain and the axillary bodies of paravertebral ganglia of spiny dogfish, *Squalus acanthias* (Euler and Fange, 1961, Nilsson, 1984, Reid *et al.*, 1995). In contrast to teleost fish NE was more abundant than EPI in these cells (Abrahamsson, 1979). Interestingly, although the tissue concentration of both NE and EPI in chromaffin tissue of elasmobranch fish appears to be greater than other ancient fishes (table 1.1) circulating levels at rest and following exposure to stress are not (table 1.2). In the more primitive group of fishes, the agnathans, large amounts of chromaffin tissue have been identified in the heart of a number of species including, the Atlantic hagfish, *Myxine glutinosa* and the river lamprey, *Lampetra fluviatilis* (Augustinsson *et al.*, 1956, Bloom *et al.*, 1961, Ostlund, 1954, Ostlund *et al.*, 1960) in addition to the PCV (Euler and Fange, 1961, Perry *et al.*, 1993, Reid *et al.*, 1995). Interestingly the catecholamines in the heart and veins of the hagfish and lampreys are different. In hagfish, NE is the major stored hormone in the atria while the remainder of the heart stores more EPI containing cells than NE (Euler and Fange, 1961, Perry *et al.*, 1993). In contrast, lampreys have more NE and EPI containing

cells in the heart and PCV respectively (Mazeaud, 1972, Reid *et al.*, 1998, Stabrovskii, 1967).

Information regarding chromaffin tissue and catecholamine secretion in acipenseriformes (paddlefish and sturgeons) is sparse. In the Mississippi paddlefish, *Polyodon spatula*, the location of chromaffin tissue was found to spread throughout the whole kidney with hyperplasia following exposure to polychlorinated and chlorine contaminated water (Gundersen *et al.*, 2000). In beluga sturgeons, *Huso huso*, chromaffin tissue has been identified in the PCV and wall of the celiac-mesenteric artery (Balashov *et al.*, 1981) and has been shown to be evenly distributed throughout the entire length of the kidney (Gallo *et al.*, 2004). The chromaffin system in the bowfin, *Amia calva*, is dispersed as yellow corpuscles under the light microscope in the head and middle portion of the kidney in association with the PCV and renal vein (Youson *et al.*, 1976). Electron microscopy studies in this species showed only one cell type where NE and EPI containing cells were not differentiated (Youson, 1976, Youson *et al.*, 1976). Chromaffin tissue in the Florida gar, *Lepisosteus platyrhincus*, is located in the swim bladder, heart and PCV. Although immunohistochemical studies demonstrated greater amounts of DBH than PNMT in all three organs the main hormone stored was EPI as was reflected in the higher circulating levels of EPI in this species (Abrahamsson *et al.*, 1981, Nilsson, 1981).

Table 1.1: Norepinephrine (NE) and epinephrine (EPI) concentrations ($ng.mg^{-1}$) in chromaffin tissue of some fish species.

Species	Organ	NE	EPI	References
Teleost				
Salmo gairdneri	Head Kidney	4.5	7.5	(Nakano and Tomlinson, 1967)
Oncorhynchus mykiss	PCV*	30	110	(Reid and Perry, 1994)
	Kidney	4	7	
Anguilla rostrata *Gadus morhua*	Head Kidney	42	84	(Hathaway and Epple, 1989)
	PCV*	14.3	38.2	(Abrahamsson and Nilsson, 1976)
Elasmobranch				
Scyliorhinus caniculua	Axillary body**	20410	14670	(Mazeaud, 1971)
Squalus acanthias	Axillary body**	2139	445	(Abrahamsson, 1979)
	Heart	0.095	0.03	(Euler and Fange, 1961)
	Kidney	19	1.9	
	Spleen	0.096	0.025	
	Intestine	0.33	0.03	
	Axillary body**	6700	3100	
Hagfish				
Myxine glutinosa	Atrium	18.0	8.1	(Euler and Fange, 1961)
	Ventricle	6.5	59.0	
	Portal Heart	58.0	3.1	
	Kidney	16.0	<0.02	
Lamprey				
Lampetra fluviatilis	Atrium	16.0	127.1	(Stabrovskii, 1967)
	Ventricle	11.6	81.0	
	Blood vessels	5.0	1.2	
Petromyzon marinus	Systemic atrium	3.4	90	(Mazeaud, 1972)
	Systemic ventricle	0.7	17	
Sturgeon				
Huso huso	PCV*	4.8	19.8	(Balashov *et al.*, 1981)
Gar				
Lepisosteus platyrhincus	PCV*	21.5	47.5	(Nilsson, 1981)

*PCV: Posterior Cardinal Vein – mainly anterior and middle regions; also known as renal portal and medial caudal veins respectively.

**Axillary body: Chromaffin located at the sympathetic chain.

1.2b Catecholamines and Stress:

As mentioned previously there are many factors known to cause a change in plasma catecholamine concentrations following exposure to stress and the increase in concentration is dependent upon the nature, duration and severity of stressors. Much of the work on fish stress physiology has focused on teleost fish and there is limited information available for the more primitive fishes. This section will discuss the various roles of catecholamines in more primitive fishes. Table 1.2 describes the circulating catecholamine concentrations in various fish species at rest and stressed states. Although, the major resting catecholamines in primitive fishes appears to be NE there is a lack of information regarding the circulating levels of catecholamines in gars and paddlefishes.

In regard to elasmobranch fish, Butler *et al.,* (1986) measured circulating levels of EPI and NE in the European lesser spotted dogfish, *Scyliorhinus canicula*, that were significantly less than measurements previously taken from other elasmobranch species (Abrahamsson and Nilsson, 1976, Butler *et al.*, 1979, Nakano and Tomlinson, 1967, Woodward, 1982). Furthermore, previous research concluded that EPI was the major circulating catecholamine in elasmobranch fish, however, it was suggested that these measurements were obtained from fish in a stressed state (Lowe *et al.*, 1995).

Agnathans not only show a difference in the catecholamine storage sites (see table 1.1) but also show large variations in innervation and catecholamine release from chromaffin tissue. Hagfish hearts in the Atlantic and Pacific hagfish, *Eptatretus stouti*, are not well innervated by the adrenergic system (Augustinsson *et al.*, 1956, Hirsch *et al.*, 1964, Johansen, 1963) and thus during stress, release more NE into the blood from well innervated kidneys, veins and the systemic heart (Fange *et al.*, 1963, Perry *et al.*, 1993). Lampreys on the other hand have well innervated hearts. Interestingly following vagal stimulation there is an increase in heart rate in two species of lampreys, the river lamprey and Arctic lamprey, *Entosphenus japonicus*, that can be abolished following treatment with nicotinic antagonists (hexamethonium or tubocurarine) prior to vagal stimulation (Augustinsson *et al.*, 1956, Lukomska and Michelso, 1972, Otorii, 1953). These effects of

catecholamines on the hagfish and lamprey's heart will be discussed in further detail in the following sections.

Table 1.2: Plasma levels of norepinephrine (NE) and epinephrine (EPI) in $ng.ml^{-1}$ at rest and stressed fishes.

Species	State	NE	EPI	References
Teleost				
Oncorhynchus mykiss	Rest	0.0013	0.0016	(Aota *et al.*, 1990)
	Acidosis	0.023	0.022	
Salmo gairdneri	Rest	1.72	0.25	(Butler *et al.*, 1986)
	Burst swimming	14.38	38.84	
Cyprinus carpio	Rest	16	1.5	(Mazeaud, 1971)
	Hypoxia	33	6.7	
Elasmobranch				
Squalus acanthias	Rest	2.0	1.5	(Opdyke *et al.*, 1983)
Scyliorhinus canicula	Rest	2.37	1.08	(Butler *et al.*, 1986)
	Burst swimming	16.32	17.64	
	Rest	48	24	(Mazeaud, 1969)
	Air exposure	138	85	
Rhinobatus typus	Rest	1.10	3.22	(Lowe *et al.*, 1995)
	Burst swimming	4.99	9.27	
Hagfish				
Myxine Glutinosa	Rest	0.30	0.07	(Perry *et al.*, 1993)
	Hypoxia	1.87	0.26	
Lamprey				
Petromyzon marinus	Rest	2.9	1.9	(Mazeaud, 1971)
	Physical disturbance	1.36	10.8	
	Rest	1.32	3.62	(Dashow *et al.*, 1982)
	Physical disturbance	31.71	6.79	
	Anesthesia	20.97	6.59	
Sturgeon				
Acipenser fulvescens	Rest	22.40	0.89	(Zubair, 2009)
	Air exposure	107.73	30.49	
Acipenser naccarii	Rest	0.73	0.95	(Randall *et al.*, 1992)
	Hypoxia	7.63	5.48	
Acipenser baerii	Hypoxia	33.16	55.14	(Maxime *et al.*, 1995)
Acipenser transmontanus	Rest	16.32	7.60	(Crocker *et al.*, 2000)
	Hypercapnia	15.48	10.96	
Bowfin				
Amia calva	Rest	2.25	1.65	(McKenzie *et al.*, 1991a)
	Acidosis	121.81	128.81	

In regard to acipenseriformes, circulating levels in sturgeons suggest a different response with respect to the nature of stressor and species. Following exposure to air for 30 seconds in lake sturgeon, the greatest relative change was seen in EPI concentration at one minute, with catecholamine levels peaking at 10 minutes post-stress (Zubair, 2009). Similarly, Crocker (2000) showed EPI as the major catecholamine in white sturgeon, *Acipenser transmontanus*, following exposure to hypercapnia, with no change in NE concentration. Conversely, there was a 159 and 77 fold increase in NE and EPI respectively following exposure of Siberian sturgeon, *Acipenser baerii*, to hypoxia (Maxime *et al.*, 1995). Randall *et al*., (1992) showed EPI levels slightly higher than NE levels at rest with a greater change in NE on exposing Adriatic sturgeon, *Acipenser naccarii*, to hypoxia for 10 minutes. In the bowfin, despite the presence of undifferentiated catecholamine cells under the electron microscope, both NE and EPI levels have been measured in the plasma. NE was the principle catecholamine at rest, although the increase in EPI was greatest following exposure to acidosis. Moreover, this change was seen in normoxic rather than hyperoxic conditions which may demonstrate that a change in partial pressure of oxygen is the major factor for secreting catecholamines (McKenzie *et al.*, 1991a). Although catecholamine levels have not been measured in gars there are studies that provide indirect evidence of catecholamine secretion in response of various stressors in this group of fishes (Smatresk and Cameron, 1982a, b, c) (see below).

1.3 – ***Catecholamine response in ancient fishes***

The response to stress in fish is entirely dependent on the nature and duration of the stressor. Furthermore, it is understood that the endocrine response can be species specific and it is widely accepted that these effects are subject to neurohumoral changes (Mazeaud *et al.*, 1977). The following section will briefly examine some of the secondary responses to a stressor in a variety of ancient fishes.

1.3a Elasmobranchs:

In mammals heart rate is maintained by specialized autorhythmic cells under the control of both parasympathetic and sympathetic nerves in addition to the adrenergic system. In elasmobranchs there is evidence of ganglionic innervation of chromaffin tissue (Opdyke *et al.*, 1983), however, there is either limited or no innervation on heart muscles (Augustinsson *et al.*, 1956, Short *et al.*, 1977). Conversely, Nilsson *et al.* (1975) described well innervated arterial vasculature in spiny and lesser spotted dogfishes and a vasoconstrictive effect through activation of α-adrenoreceptors following administration of both EPI and NE which has been confirmed in a number of other elasmobranch species (Capra and Satchell, 1977b, Kent and Peirce, 1978, Opdyke *et al.*, 1982). Despite the lack of direct innervation in the elasmobranch heart there is a pronounced tachycardia following an increase in catecholamines (Bernier *et al.*, 1999, Capra and Satchell, 1977a, Farrell *et al.*, 1986). In the coronary arteries of the shortfin mako shark, *Isurus oxyrinchus* and rough skate, *Raja nasuta*, β-adrenoreceptors are abundant and will induce dilation following addition of catecholamines (Farrell and Davie, 1991a, b). Both the tachycardic effects and vasodilatory effects in coronary arteries are consistent with the observed effects of the adrenergic system in teleost fishes and mammals (Farrell *et al.*, 1989, Hamilton and Feigl, 1976, Ross, 1976). Both α and β adrenoceptors have been identified on the vasculature of the elasmobranch gill, however, isolated perfusion experiments on gill vasculature with catecholamines have shown a decrease in ventilatory resistance in the spiny dogfish and western shovelnose stingaree, *Trygonoptera mucosa*, (Davies and Rankin, 1973, Donald, 1988, Metcalfe and Butler, 1984) an increase in ventilatory resistance in spiny dogfish and sparsely spotted stingaree, *Urolophus paucimaculatus,* (Capra and Satchell, 1974, Donald, 1988) and dose dependent vasoconstrictive and vasodilatory effects using NE in blacktip reef shark, *Carcharhinus melanopterus* (Chopin and Bennett, 1995). Despite evidence of ganglionic innervation of chromaffin tissue in elasmobranchs (Opdyke *et al.*, 1983) there is no effect of adrenergic stimulation on red blood cells or a change in oxygen carrying capacity of blood following a stressor such as hypoxia (Lowe *et al.*, 1995, Perry and Gilmour, 1996, Tufts and Randall, 1989, Wood *et al.*, 1994).

Table 1.3: Various cardiovascular parameters in resting and stressed primitive fishes including, Cardiac output (CO) in $ml.kg^{-1}.min^{-1}$, Heart rate (HR) in beats per minute (BPM), Ventral aorta pressure (Pva) in kPa, Dorsal aorta pressure (Pda) in kPa and Systemic resistance (RS) in $Pa.ml^{-1}.kg^{-1}.ml^{-1}$. EPI, epinephrine.

Species	State	CO	HR	Pva	Pda	RS	References
Teleost							
Gadus morhua	Rest	17.3	43.2	4.9	3.2	188	(Axelsson and Nilsson, 1986)
	Swim Exercise	25.4	51.2	6.2	4.0	174	
Anguilla australis	Rest	6.8	47.7	5.26	3.03		(Davie and Forster, 1980)
	Swim Exercise	6.4	49.2	6.24	2.91		
Elasmobranch							
Raja rhina	Rest	21.1					(Satchell *et al.*, 1970)
	Swim Exercise	23.3					
Scyliorhinus canicula	Rest	43.7	35.3	5.37	4.41		(Taylor *et al.*, 1977)
	Hypoxia	26.9	16.2	4.45	3.59		
Scyliorhinus stellaris	Rest	52.5	41	3.36	2.53	46.7	(Piiper *et al.*, 1977)
	Swim Excercise	89.2	48	3.40	2.42	26.6	
Hagfish							
Myxime glutinosa	Rest	8.7	22.3	1.04	0.77	113	(Axelsson *et al.*, 1990)
	EPI Injection	25	24				
Eptatretus cirrhatus	Rest		25.8	1.44	1.07		(Forster *et al.*, 1988)
	Swim Exercise		28.8	1.55	1.00		
Lamprey							
Entosphenus tridentatus	Rest	32			2.5 – 4.3		(Johansen *et al.*, 1973)
Sturgeon							
Acipenser transmontanus	Rest	36.1	48.0	2.92*		87.99	(Crocker *et al.*, 2000)
	Hypercapnia	47.3	51.8	3.01*		70.39	
Acipenser naccarii	Rest		63.5	2.7*			(McKenzie *et al.*, 1995)
	NE Injection		69.3	4.7*			
Bowfin							
Amia calva	Rest		27.2		3.27		(Butler *et al.*, 1995)
Gar							
Lepisosteus oculatus	Rest	31	32.9	2.71	3.17		(Smatresk and Cameron, 1982a)
	Hypoxia	40.5	35.9	3.01	2.92		

* Pva and Pda were not measured, and thus mean arterial pressure (kPa) is shown.

1.3b Agnathans:

Hagfish are known to have a very high blood volume and comparatively low heart rate and have the lowest recorded aortic blood pressure among vertebrates (Forster, 1989, Forster *et al.*, 1991, McCarthy and Conte, 1966) (also see table 1.3). That said cardiac output is comparable to elasmobranchs and some teleost fishes (Axelsson *et al.*, 1990, Forster, 1989), demonstrating a 'low pressure, moderate flow system' (Forster *et al.*, 1991). As the hagfish heart lacks vagal and adrenergic innervation (Augustinsson *et al.*, 1956, Nilsson, 1983), there is no bradycardia following exposure to stress, but they do respond well to a stressful situation (table 1.3). In one study conducted by Forster *et al.* (1992) a 40% increase in cardiac output of broadgilled hagfish, *Eptatretus cirrhatus*, was observed on exposure to hypoxia. This was followed by a 160% increase in cardiac output during the recovery period. Furthermore, there was a fall in heart rate following administration of β-adrenoceptor blocker propanolol indicating adrenergic control of heart rate in a resting condition. Likewise, Foster *et al.* (2008) demonstrated β-adrenoceptor mediated vasodilation while α-adrenoceptor mediated vasoconstriction was observed in vessels of broadgilled hagfish using adrenergic agonists, phenylephrine and isopernaline.

1.3c Acipenserids:

In sturgeons there is both α and β innervations of smooth muscle vasculature and cardiac muscle respectively. Following exposure to hypoxia, white and Adriatic sturgeon exhibited an initial tachycardia followed by marked bradycardia with a rise in mean arterial pressure (Crocker *et al.*, 2000, McKenzie *et al.*, 1995). This sequence of events could be explained by the positive ionotropic and chronotropic effects of catecholamines on activating β-adrenoceptors within cardiac muscle and vasoconstrictive effects on vascular smooth muscle through activation of α-adrenoceptors (Wood and Shelton, 1975, 1980). Conversely, bradycardia could be under the influence of vagal nerve (parasympathetic) stimulation as a compensatory mechanism of increased mean arterial pressure (Hughes, 1973). McKenzie *et al*., (1995) demonstrated the latter, as prior treatment with both the β-adrenoceptor blocker propranolol and the muscarinic receptor

blocker atropine, inhibited the observed bradycardia following hypoxia. Although sturgeons have relatively high concentrations of circulating catecholamines, compared to teleost fish, they demonstrate less of a change in magnitude in cardiovascular parameters. This disproportionality is thought to be the result of a decreased sensitivity to catecholamines in sturgeons, however, this requires further study.

1.3d Holostei:

Currently there is no data providing evidence of direct effects of catecholamines on cardiovascular or any other system of gars. However, chromaffin tissue has been identified in the swim bladder, heart and PCV (see above), and there are well developed adrenergic nerve endings in the heart blood vessels in addition to excitatory cholinergic endings in the swim bladder (Abrahamsson *et al.*, 1981, Nilsson, 1981). Furthermore, various stress factors including hypoxia and changes in environmental temperature and osmolality, have been shown to increase heart rate, dorsal and ventral aortic pressures, air breathing frequency and decreases in blood pH (De Roth, 1973, McCormack, 1967, Saksena, 1975a, b, Smatresk and Cameron, 1982a, b, c). These effects suggest the involvement of catecholamines, however, further research is required to confirm this.

In bowfin, McKenzie *et al.*, (1991a) showed an increase in cardiovascular and respiratory variables following infusion of NE and EPI in the blood. Interestingly, they found a positive effect on gill ventilation but no effect on the air breathing of these fishes. In another experiment denervation of the gills and pseudobranch ablation in this species, (McKenzie *et al.*, 1991b) resulted in an increase in heart rate and blood pressure but had no effect on gill ventilation, suggestive of a direct effect of circulating catecholamines on the measured cardiovascular variables. Later Butler *et al.*, (1995) confirmed that these effects were under the influence of α-adrenoceptors in peripheral vasculature by observing hypotension following infusion of the α-adrenoceptor blocker, phentolamine. In conclusion, these effects on peripheral circulation are similar to the teleost, elasmobranchs and Acipenserids (see above).

Chapter 2: The Influence of Social Environment on the Catecholamine Stress Response in Juvenile Lake Sturgeon

2.1 – ***Introduction***

Social factors have been shown to have a significant influence on the stress axis of teleost fish both at the behavioral and molecular levels (Currie *et al.*, 2010, Gilmour *et al.*, 2005, Roberge *et al.*, 2008). This topic has gained popularity in recent years, however, most of the research has focused on the influence of the corticosteroids rather than the catecholamines. Furthermore, our understanding of social factors influencing the general physiology of ancient fishes is very limited. It is widely accepted that many teleost fish exhibit aggressive behavior with a high mortality rate showing classical dominant versus subordinate behavior when they are allowed to live at high stocking densities (Pickering, 1992, Schreck, 1981). This is especially true in laboratory conditions where subordinate groups are unable to seek refuge from their dominant competitors (Overli *et al.*, 1999a). Those individuals that have higher cortisol levels prior to social interaction are less likely to exhibit dominant behavior and more likely to be the subordinate individuals (Gilmour *et al.*, 2005, Sloman *et al.*, 2001).

During the establishment of a social hierarchy in rainbow trout social interaction will result in the creation of dominant and subordinate fish. The subordinate fish experience significant stress resulting in an increase in circulating levels of cortisol that is much higher than seen in the dominant fish (Laidley and Leatherland, 1988, Schreck, 1981, Sloman *et al.*, 2002). In addition the subordinate fish experience significant changes in respiratory rate, leukocyte count and plasma glucose alongside a decrease in glycogen stores, body weight and reproductive capacity, likely due to a decrease in circulating concentrations of plasma testosterone and 11-ketotestosterone (Ejike and Schreck, 1980, Elofsson *et al.*, 2000, Fox *et al.*, 1997, Peters *et al.*, 1980, Peters *et al.*, 1988). In experimental studies there is often considerable variability in these parameters. This could be the result of different susceptibilities of individuals to respond to a given stressor or discrete differences in the magnitude or type of stressors (Peters *et al.*, 1980,

Peters *et al.*, 1988). Following the initial conflict and establishment of a hierarchy the situation in the dominant fish more closely resembles unstressed individuals.

Initially, a decrease in body weight in rainbow trout was believed to be due to avoidance of food in subordinate groups (Rubenstein, 1981). However, many researchers have provided alternative explanations including higher activity levels leading to higher metabolic rates in dominant fish (Carline and Hall, 1973, Kalleberg, 1958, Newman, 1956) and undefined psychological factors (Jobling and Wandsvik, 1983). Overall this causes dominant fish to grow faster as compared to their subordinate companions (Abbott and Dill, 1989, Metcalfe, 1986, Sloman *et al.*, 2000). Peters *et al.*, (1988) explained the effect on defense mechanisms of rainbow trout by intramuscular injection of bacteria, *Aeromonas hydrophila*, in severly socially stressed fish. Bacteria were able to overcome defensive mechanisms in subordinate fishes more easily than in the dominant individuals. Moreover, dominant fish did not show hematopoietic invasion which was observed in the subordinate group. These findings can be explained with the results of numerous other studies in fish showing hypertrophy in granulocytes with progressive degeneration, lymphocytopenia and suppression of B cell activity (Peters and Schwarzer, 1985, Pickering *et al.*, 1982, Pickford *et al.*, 1971a, b, Tripp *et al.*, 1987, Wedemeyer *et al.*, 1983) when individuals are exposed to stressors. Pickford et al., (1971a, b) named these effects as the 'Leucocyte Stress Syndrome' and demonstrated a significant role of both catecholamines and cortisol in the development of the syndrome in the killifish, *Fundulus heteroclitus*.

Interestingly dominant status does not always confer a reduced stress response in fish. Measurement of plasma cortisol levels in subordinate and dominant arctic char, *Salvelinus alpinus*, following acute handling revealed a much greater response in the dominant indivduals as opposed to the subordinate individuals (Overli *et al.*, 1999b). Sloman *et al.*, (2002) suggested this phenomenon as a negative feedback mechanism of chronically increased cortisol to the HPI axis in the subordinate individuals. She stimulated interrenal tissue of rainbow trout *in vitro* by ACTH and found a lower rate of cortisol secretion in subordinate individuals compared to dominant individuals despite the higher plasma cortisol levels in the subordinate fish. In socially stressed fish various

researchers have demonstrated an increase in corticotrophin releasing hormone *in vivo* in addition to plasma ACTH and its precursor pro-opiomelanocortin in the pituitary (Doyon *et al.*, 2003, Hoglund *et al.*, 2000, Winberg and Lepage, 1998). This adds further support to negative feedback regulation leading to a reduced increase in cortisol in subordinate fish following a stressor.

To date the only study that has examined a role for catecholamines in social stress provided results that were equivocal. That is perfusion of acetyl choline in the PCV of both dominant and subordinate rainbow trout stimulated catecholamine synthesis and release to the same extent in both groups (Sloman *et al.*, 2002).There is currently no literature available on the potential role of social stress and catecholamines *in vivo*.

Acipenserids are thought to have evolved around 184 million years ago (Peng *et al.*, 2007). Along with many other ancient species they are highly social animals that are rarely found in isolation in the wild, particularly at the juvenile stage (Barth *et al.*, 2011, Benson *et al.*, 2007, Rochard *et al.*, 2001). There are a number of benefits associated with group living, including increased mating opportunities, decreased vigilance and increased growth rates (Roberts, 1996, Shapiro *et al.*, 1993, Stirling, 1977). Georgiadis *et al.* (2000), described the indirect effect of dominant behavior on the stress axis in white sturgeon, by isolating subordinate individuals from dominant fish there was a significant change in mortality and recovery rates. In juvenile lake sturgeon no dominant behaviour was observed, however, fish maintained in isolation were found to have a longer lasting cortisol stress response as opposed to those maintained in groups suggestive of a social influence on the stress response in lake sturgeon (Allen *et al.*, 2009). Interestingly , in both Atlantic sturgeon, *Acipenser oxyrinchus*, and short nose sturgeon, *Acipenser brevirostrum*, aggression and dominance were observed in a limited foraging situation (Kynard and Horgan, 2002) although this study did not examine the stress response in these species.

In sturgeons the cortisol stress response is attenuated in comparison to teleost fish (Wendelaar-Bonga, 1997). However, this does not necessarily equate to a reduced physiological response of the whole organism and does not include examination of the

catecholamine component of the stress response indeed our understanding of the release of catecholamines following exposure to an acute stressor is not well understood in sturgeon. In the present study I have expanded on the research conducted by Allen *et al.*, (2009) and studied the possible influence of stress on catecholaminergic axis of lake sturgeon and whether maintaining juvenile lake sturgeon in isolation or groups influences the magnitude and duration of the catecholamine stress response.

2.2 – ***Materials and Methods***

Juvenile lake sturgeons, sex undetermined, were divided into two experimental groups. In one group fish were maintained in isolation (mean weight of 237.97g ± 3.76) and in the second group fish were held in a group of five fish (mean weight of 243.09g ± 7.71) for a minimum of two weeks. Treatment groups were held in identical conditions in flow through aquaria at 16 ± 1°C with a 16h:8h day:night cycle. Following acclimation a single fish in each treatment was anaesthetized in 200ppm tricaine methanesulphonate (MS222) (Syndel Laboratories Ltd. Qualicum beach, BC, Canada) buffered with an equivalent mass of sodium bicarbonate. When opercular movement had ceased fish were removed and placed ventral side up on a specially designed surgical V shaped board. Aerated water containing 100ppm MS222 was constantly irrigated across the gills during surgical insertion of a PE 50 cannula (Fisher Scientific, Fair lawn, NJ, USA) in the caudal sinus of the fish. The cannula was secured in place with at least 2 anchoring sutures in the tail region of the fish. Fish were then returned to their experimental tank and allowed to recover for a minimum of 48 h prior to the onset of the experiment.

All fish in each treatment, isolation or grouped, were exposed to a stressor by removing the external standpipe in their holding tank to allow the water to totally drain from the tank. Fish were then left air exposed for one minute, the standpipe was then replaced and the tank allowed to re-fill. Blood samples were taken at time, 0 (one hour before the stressor), then 1, 5, 10, 20 and 120 minutes post-stressor. Approximately 600µl of blood was taken at each sampling time point. Haematocrit was immediately measured (CritSpin; Micro-haematocrit centrifuge) and the remaining whole blood was centrifuged

at 13,000g for 3 minutes at 4°C. The plasma was removed for further analysis (see below) and the red blood cells were resuspended in equivalent volumes of Sturgeon Ringers solution (126 mM NaCl, 2.2 mM KCl, 0.45 mM $CaCl_2$, 3 mM $MgCl_2$, 4.6 mM Na_2HPO_4 and 0.2 mM KH_2PO_4 with a final adjusted pH of 7.6) (Dettlaff *et al.*, 1993, LeBreton and Beamish, 1998) and re-injected into the animal to avoid excessive disturbance of body fluid volume throughout the experimental period.

Catecholamines were extracted from 300µL of plasma using the Acid Washed Alumina technique as outlined by Ganhao *et al.* (1991). Measurement of NE and EPI was conducted by liquid chromatography and using ChromGraph software (Epsilon system, BASi, West Lafayette, IN 47906 USA) as described previously by Zubair (2009). $100ng.ml^{-1}$ of dihydroxybenzylamine was used as an internal standard. All the chemicals for making external and internal standards (EPI, NE and dihydroxybenzylamine) were purchased from Sigma-Aldrich, Inc. (St. Louis, USA) The remaining plasma was then frozen and stored at -20°C until further analysis. The following parameters were assessed to provide further indication of the impact of air exposure on secondary stress parameters. Osmolality was measured by vapor pressure (Wescor, Logan, Utah, USA). Glucose was assessed using a commercial assay (Wako, USA) and measured spectrophotometrically (Power wave XS2, BioTek, Winooski, Vermont, 05404-0998, USA). In addition pH was measured using an AB15 Accumet basic pH meter (Fisher Scientific, Fair lawn, NJ, USA) and bicarbonate ions as a further measure of blood acid/base balance were measured using the total CO_2 method with a Corning 965 carbon dioxide analyzer.

Statistical analysis:

Significant differences in all measured values between treatment groups (group vs isolated) at each time point were analysed using an unpaired Student's t-test. Statistical differences within treatment groups from time zero values for each of the measured variables was analysed using one way analysis of variance followed by a Bonferroni's post-hoc text. Data are expressed as a mean ± standard error (S.E) and significance was accepted when $p< 0.05$. All statistical analyses were conducted using GraphPad Prism

software, v: 5.01 (GraphPad Software , Inc., CA, USA) and graphs were prepared using Sigma plot software, v: 10.0 (Systat, San Jose, CA, USA).

2.3 – ***Results***

Figure 2.1 illustrates the effect of air exposure on the plasma concentration of NE and EPI respectively. There was no significant difference between treatments at any of the time points tested. However, profiles of both NE and EPI were different between the isolated and grouped treatments where baseline concentrations of NE was higher than EPI and furthermore the relative increase in EPI was greater than NE following air exposure. Plasma concentration of NE in the isolated treatment (n=6) was significantly higher than baseline values 1 minute post-stress, this increase remained higher than baseline values, but not significantly so, until the 120 minute post-stress sampling time point. In the grouped treatments (n=4) although reported values followed a similar trend as described for the isolated treatment there was no significant difference in plasma NE concentrations between any time point (Figure 2.1A).

Plasma concentration of EPI was significantly higher 1 minute post-stress in both the isolated and grouped treatments. Values then followed a similar trend in both treatment groups where at 5, 10 and 20 minutes post-stress plasma EPI concentrations were higher than time zero values but not significantly so. At 120 minutes post-stress values in the grouped treatment (n=4) were significantly lower than the measured peak value 1 minute post-stress, however, in the isolated treatment (n=6) while the measured value was lower this was not significantly lower than the peak value in that treatment group (Figure 2.1B).

Plasma glucose concentration followed a similar trend for both the isolated (n=15) and grouped (n=10) treatments where concentrations were significantly higher than time zero 5 minutes post-stress and remained there for the duration of the experiment. Between treatments analysis for plasma glucose revealed a significantly higher plasma glucose concentration in the isolated treatment compared to the grouped treatment at 1

minute post-stress (Figure 2.2A). A similar trend within each treatment was also observed for plasma osmolality. In the isolated (n=15) treatment plasma osmolality was significantly different from time zero values 10 minutes post-stress and returned to baseline values for this treatment at 20 and 120 minutes post-stress. In the grouped (n=10) treatment plasma osmolality was significantly higher at 5, 10 and 20 minutes post-stress and returned to values similar to time zero of isolated group at 120 minutes post-stress but not significantly so (Figure 2.2B).

Plasma pH was not significantly different between time points within the isolated (n=15) treatment group. However, in the grouped (n=10) treatment plasma pH was significantly lower than time zero values at 10 and 20 minutes post-stress (Figure 2.3A). Similarly plasma bicarbonate concentration in the grouped (n=13) fish was significantly lower at 10 and 20 minutes post-stress, however similar trends were also observed in the isolated (n=10) treatment group (Figure 2.3B). Interestingly, both pH and bicarbonate concentration decreased more in fish in the grouped treatment compared with the isolated treatment (table 2.1).

Table 2.1: Percent decrease in pH and bicarbonate concentration at 10 and 20 minutes post-stress from baseline values.

	Minutes post-stress	**Group (%)**	**Isolated (%)**
Bicarbonate Concentration	10	10.06*	7.34*
	20	13.27*	9.87*
pH	10	2.41*	0.24
	20	2.41*	0.36

* Significant decrease from baseline value ($p \leq 0.05$).

Finally plasma haematocrit remained constant in both isolated (n=12) and grouped (n=14) treatments and did not show any significant difference between treatments at any point time (Figure 2.4).

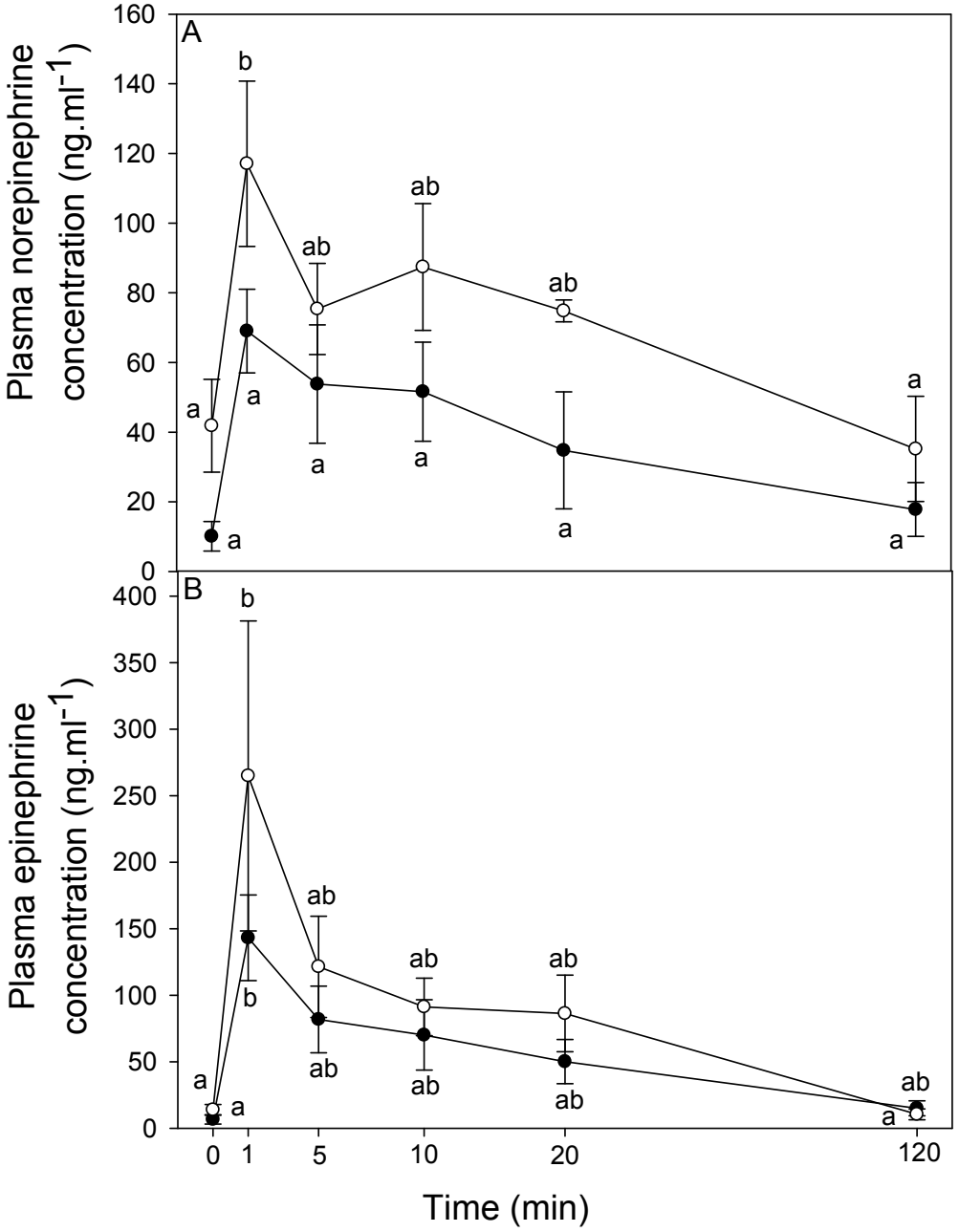


Figure 2.1: Plasma concentration of Norepinephrine (A) and Epinephrine (B) following a one minute exposure to air in isolated (n=6, open circles) and grouped (n=4, closed circles) juvenile lake sturgeon. Different letters denote significant differences between time points within treatments with letters above the lines representing the isolated treatment and letters below the lines representing the grouped treatments. Data are expressed as a mean ± SEM and significance was accepted when $p \leq 0.05$.

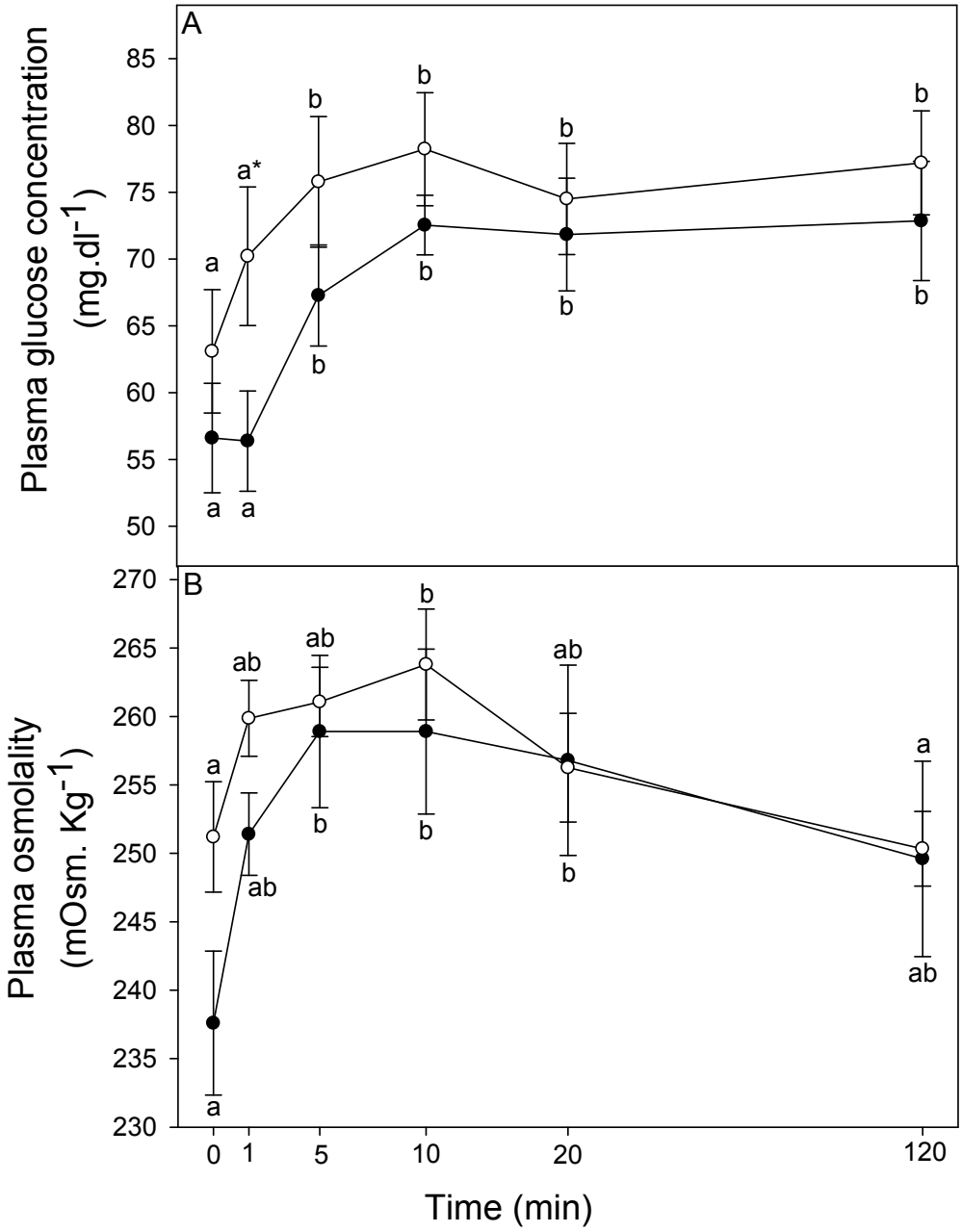


*Figure 2.2: Plasma concentration of glucose (A) and plasma osmolality (B) following a one minute exposure to air in isolated (n=15, open circles) and grouped (n=10, closed circles) juvenile lake sturgeon. Different letters denote significant differences between time points within treatments with letters above the lines representing the isolated treatment and letters below the lines representing the grouped treatments. Data are expressed as a mean ± SEM and significance was accepted when $p \leq 0.05$. * $p<0.05$, represents a significant difference between treatments at the same time point.*

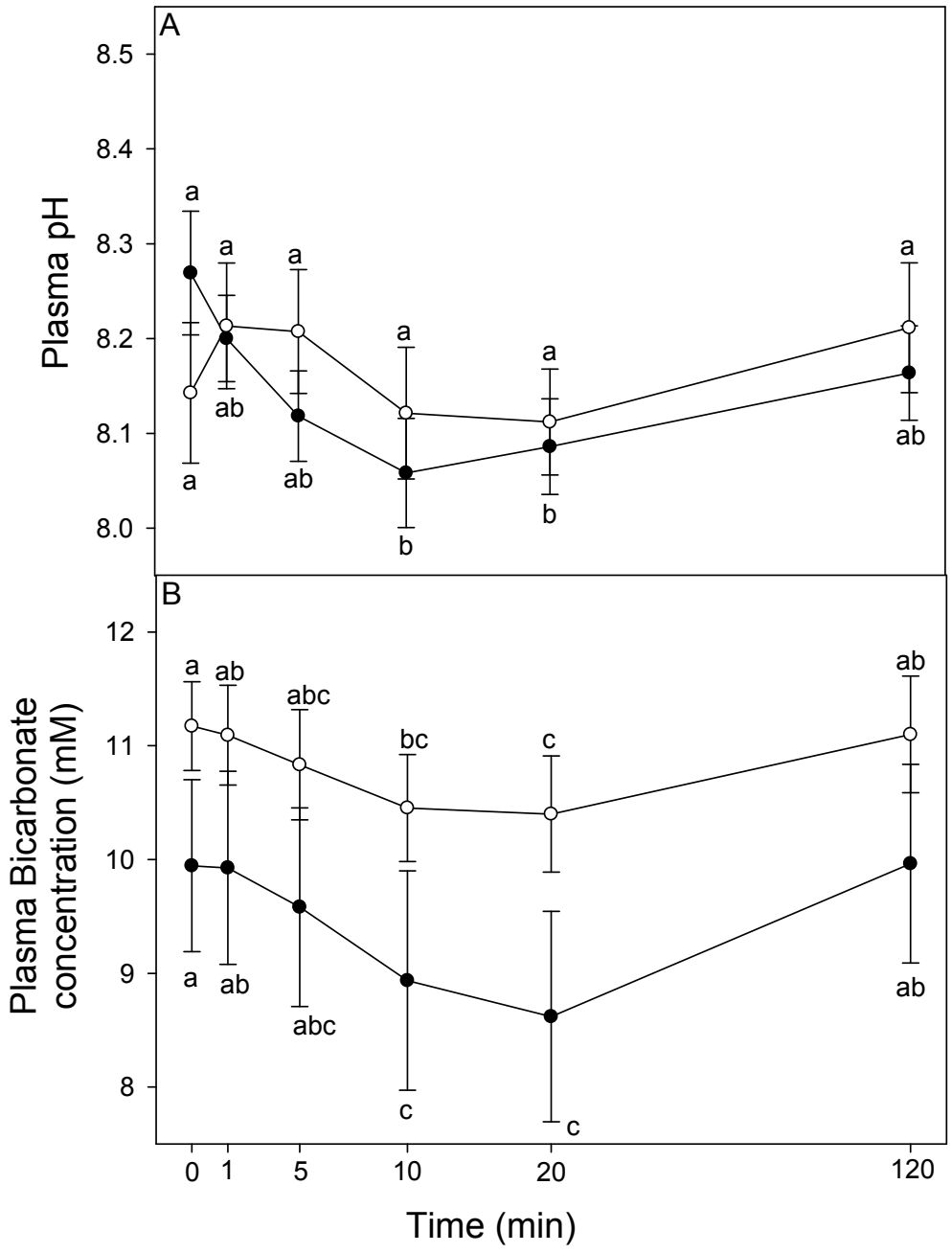


Figure 2.3: Plasma pH (A) (isolated, n=15; group, n=10) and plasma concentration of bicarbonate (B) (isolated, n=10; group, n=13) following a one minute exposure to air in isolated (open circles) and grouped (closed circles) juvenile lake sturgeon. Different letters denote significant differences between time points within treatments with letters above the lines representing the isolated treatment and letters below the lines representing the grouped treatments. Data are expressed as a mean ± SEM and significance was accepted when $p \leq 0.05$.

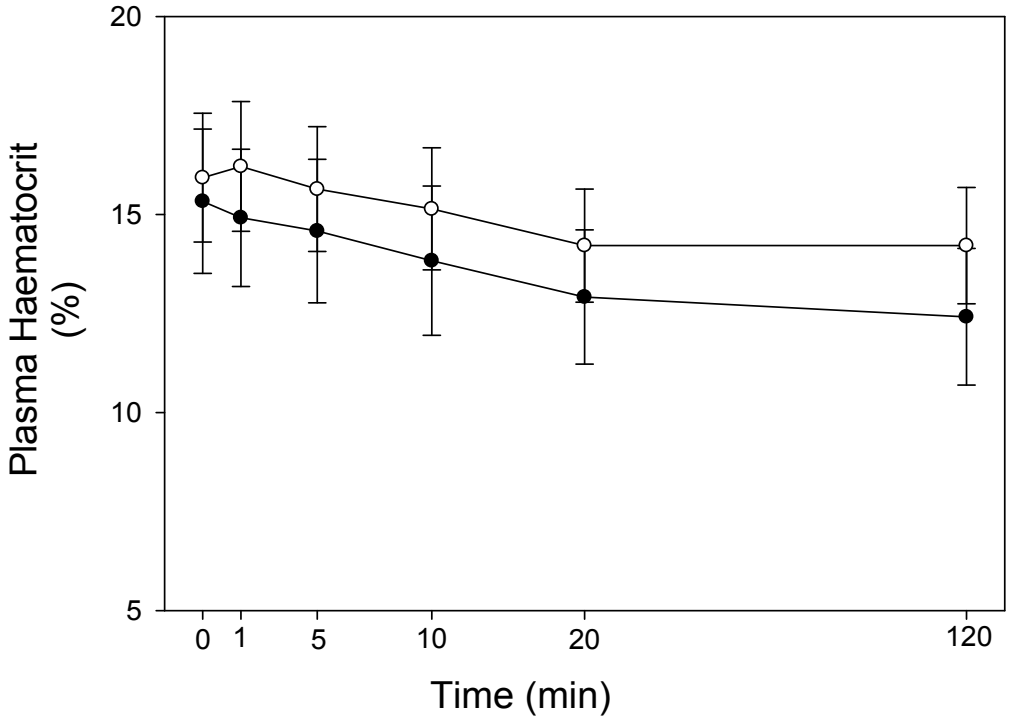


Figure 2.4: Plasma haematocrit expressed as a percentage of packed red cells following a one minute exposure to air in isolated (n=12, open circles) and grouped (n=14, closed circles) juvenile lake sturgeon. Data are expressed as a mean ± SEM .

2.4 – ***Discussion***

In the present study under baseline conditions, that is the time zero sampling period, NE dominated particularly in the isolated treatment groups. However, within one minute of the stressor circulating concentrations of EPI were between 2 and 3 fold greater. This response was consistent with previously reported results in juvenile lake sturgeon following a 30 second aerial exposure (Zubair, 2009). Furthermore, between treatment analysis in the previous study demonstrated significant difference between fish in isolation and groups at 1, 5, 10 and 20 minutes post-stress, however, between treatment analysis was not significantly different at any time point for either NE or EPI in isolated and grouped fish. This was likely a function of the low n values in the present study and further experimentation may yield significant differences in the measured values of both EPI and NE between treatment groups.

In rainbow trout a 7 day exposure to exhaustive exercise resulted in a down regulation of β-adrenoceptors (Perry *et al.*, 1996) , furthermore, following repeated exposure of a similar stress in rainbow trout there was a decrease in catecholamine release from chromaffin tissue following treatment with the cholinergic agonist carbachol (Reid *et al.*, 1994). These studies suggested a habituation of the catecholamine stress response to repeated physical stressors in teleost fish. However, in the green sturgeon, *Acipenser medirostris*, exposure to a repeated stressor did not result in habituation (Lankford *et al.*, 2005). Interestingly, repeated stress in the context of sociality influences the cortisol stress response but may have no effect on the catecholamine stress response. Following a one month acclimation period, measurement of cortisol and catecholamines in dominant and subordinate fish demonstrated a significant increase in cortisol in socially stressed fish with no change in the cateholamine response (Sloman *et al.*, 2002). While current evidence suggests that lake sturgeon do not establish dominance hierarchies in the same way as rainbow trout it has been shown that social isolation in the lake sturgeon following a similar experimental protocol as the present study resulted in a prolonged cortisol stress response in juvenile lake sturgeon (Allen *et al.,* 2009) and the present study suggests that the isolation may not have an effect on the catecholamine stress response.

Table 2.2: Peak plasma glucose concentration ($mg.dl^{-1}$) following a stressful event in various teleost and sturgeons.

Species	State	Glucose	References
Teleost			
Pleuronectes platessa	Rest	22.0	(Bourne, 1986)
	Capture and captivity	87.92	
Salmo gairdneri	Rest	49.4	(Swift, 1981)
	Sub-lethal Phenol exposure	124.2	
Scomber scombrus	Rest	67.1	(Swift, 1983)
	Handling	109.8	
Sander vitreus	Rest	108	(Killen *et al.*, 2003)
	Angling and transport challenge	450	
Morone saxatilis	Rest	144	(Mazik *et al.*, 1991)
	Angling and transport challenge	324	
Plectropomus leopardus	Rest	36	(Frisch and Anderson, 2000)
	Angling and transport challenge	136.08	
Salvelinus fontinalis	Rest	122.4	(Biron and Benfey, 1994)
	Handling	169.2	
Sturgeon			
Acipenser medirostris	Rest	65.88	(Warren *et al.*, 2004)
	Acid infusion	71.64	
Scaphirhynchus albus	Rest	63	(Barton *et al.*, 2000)
	30s Handling	59.6	
Acipenser brevirostrum	Rest	72	(Beyea *et al.*, 2005)
	Chasing	45	
Acipenser naccarii	Rest	46.8	(Cataldi *et al.*, 1998)
	Multiple stress*	61.2	
Acipenser fluvescence	Rest	63.14	Present study
	Air exposure	77.57	
	Rest	56.8	Allen *et al.*, (2009)
	Air exposure	76.5	
	Rest	99.0	(Baker *et al.*, 2008)
	Capture and handling	165.6	

*Anesthetized + low temperature + crowding + prolonged handling

Chronic stress maintains fish homeostasis through negative feedback mechanisms on various indicators of stress (Wendelaar-Bonga, 1997). Stimulus of hepatic glycogenolysis and gluconeogenesis are two recognized routes by which both cortisol and catecholamines can increase circulating levels of glucose in fish (Fabbri *et al.*, 1998, Perry *et al.*, 1988, Wendelaar-Bonga, 1997, Wright *et al.*, 1989). The present study has shown an identical increase in plasma glucose concentrations for both the isolated and grouped treatments following a stressor. Furthermore, while not significantly different,

glucose concentration was higher in the isolated treatment group at every time point which, may as previously indicated, be a measure of chronic stress in the isolated group (Allen *et al.*, 2009). Interestingly, exposure to a variety of stressors in sturgeons has failed to elicit a substantial increase in circulating glucose concentrations (table 2.2). However in lake sturgeon, Baker *et al.* (2008) showed a significant increase in plasma glucose concentrations following acute capture and handling while Allen *et al.* (2009) showed a significant increase in glucose concentrations in grouped sturgeon at 20 minutes that returned to baseline concentrations 120 minutes post-stress. Furthermore, in this and other studies the peak measured values for glucose in the circulation appears to be higher in lake sturgeon when compared to other sturgeon species but as expected lower than teleost fish (table 2.2). Therefore, although lake sturgeon may have an attenuated glucose response compared to teleost fish they appear to be able to mobilize glucose to a greater extent compared to other sturgeon species. Possible explanations could include a more developed glucose synthetic pathway or reduced negative feedback mechanisms in comparison to other sturgeon species. The later explanation is unlikely, nonetheless the glucose responsiveness in this species of sturgeon presents an interesting comparison that warrants further investigation.

Plasma osmolality in the present study showed a similar trend as previously reported by Allen *et. al.* (2009) and Baker *et al.* (2008) in lake sturgeon. Fishes in both treatments showed raised values 10 minutes post-stress and returned to baseline with the exception of grouped fish which remain elevated at 120 minutes post-stress. Although ions were not measured in the present study in a similarly designed experiment Allen *et. al.* (2009) found transient increases in Na^+, Cl^-, Br^-, PO_4^{3-} and SO_4^{2-} with decreases in K^+ and Mg^{2+}. Similarly Baker *et. al.* (2008) found increases in Cl^- immediately following capture and handling. Along with glucose, osmolality may also represent unique characteristics of these species as stress in other sturgeon speices demonstrated either comparatively less or no significant change (Baker *et al.*, 2005, Beyea *et al.*, 2005, Cataldi *et al.*, 1998, Gisbert *et al.*, 2004). Allen *et al.* (2009) hypothesized this increase in osmolality was due to decrease in plasma volume, however, the lack of change in haematocrit does not support this hypothesis. Further studies are required to determine the discrepancy between haematocrit and plasma osmolality results.

Plasma pH and bicarbonate concentration demonstrated similar trends in all treatments with the exception of pH in the isolated treatment. Interestingly the extent of pH and bicarbonate decline was greater in the grouped treatment than the isolated treatment. The influence of stress and acid base balance is well recognized in fish. In rainbow trout β-adrenoceptors has been identified which on stimulation increase Na^+/H^+ pump activity in red blood cells (Nikinmaa, 1992) resulting in intra-cellular alkalization and plasma acidification (Primmett *et al.*, 1986). Water moves into the intracellular space with Na^+ resulting in red cell swelling while alkalization decreases the hemoglobin-oxygen binding capacity of red blood cells resulting in an increase of partial pressure of oxygen (Wendelaar-Bonga, 1997). Similar effects have also been observed in the American eel, *Anguilla rostrata*, but β-adrenoceptors were found to be at a much lower abundance than was observed in the rainbow trout (Perry and Reid, 1992). No such effect was identified in elasmobranches (see section 1.3a) and currently there is no study demonstrating the evidence of adrenoceptors in red blood cells of any sturgeon species. An additional result of blood acidification following stress is an increased ventilatory rate in fishes. In the grouped treatment this would lead to a greater rate of oxygen consumption from the environment than the isolated treatment based on the demand of oxygen uptake from the equivalent sized tanks used in the present study. This may explain the greater decrease in plasma pH and bicarbonate concentration in the grouped treatment.

Following exposure to stress in a variety of teleost species there is an increase in haematocrit that leads to an increase in oxygen carrying capacity of the blood (Davison *et al.*, 1995, Franklin *et al.*, 1993, Witeska *et al.*, 2010, Witters *et al.*, 1990). This effect has been shown to be the result of increased catecholamines stimulating the production and release of red blood cells from the spleen (Kita and Itazawa, 1994, Sandblom *et al.*, 2010). In the present study although there was a slight insignificant increase of haematocrit 1 minute post-stress, overall there was no significant increase as demonstrated in teleost fish. However, similar results have been reported for both juvenile and adult lake sturgeon post-stress (Allen *et al.*, 2009, Baker *et al.*, 2005, Zubair, 2009). This lack of increase in haematocrit may be the result of reduced innervation

and/or adrenoceptors in the spleen or may be the result of an as yet not fully developed stress response.

Chapter 3: Morphology of Chromaffin and Renal Tissue in Pro-Larval Lake Sturgeon and Influence of Environmental Factors in its Development

*3.1 – **Introduction***

Much of the research on embryology and larval development in fish has focused on telesot fishes and so this provides an excellent comparison to the developmental aspects examined in the present study. The following section will examine the development of the teleost circulatory system related to renal function and briefly describe the varied morphology of chromaffin tissue in comparison to what is known in more ancient fishes.

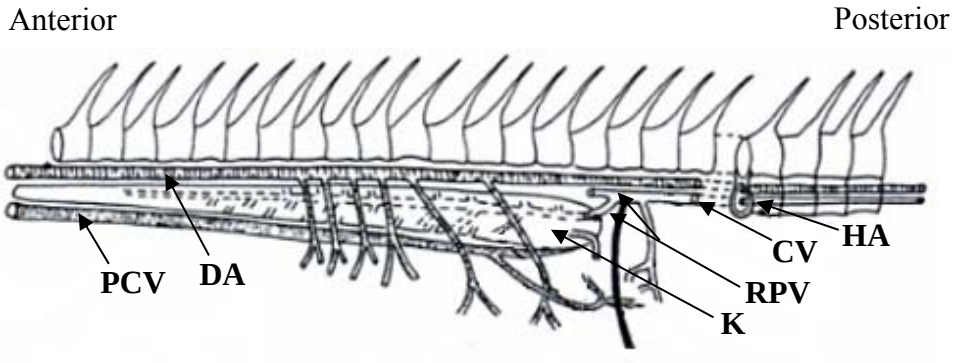


Figure 3.1: Main blood vessels related to the kidney in a typical teloest fish with the cephalic end to the left and caudal to the right, dorsal on the top and ventral on the bottom. CV, cardinal vein; DA, dorsal aorta; HA, hemal arch; K, kidney; PCV, posterior cardinal vein; RPV, renal portal vein. Figure adapted from Satchell (1991).

Figure 3.1 shows the kidney and major vessels in a typical teleost fish. The dorsal aorta, receives oxygenated blood from the gills, runs the entire length of the kidneys and rests on the ventral aspect of vertebral column. In the trunk region the dorsal aorta supplies blood to the bilateral kidneys, myotomes and fins and continues to the haemal arch (Satchell, 1991). After emerging from haemal arch the Cardinal vein runs ventral to the dorsal aorta and divides at the caudal end of kidneys into the right and left renal portal

veins. The bilateral renal portal veins run within the renal tissue draining blood into the PCVs. The PCV runs on each side of the midline either partially or completely embedded

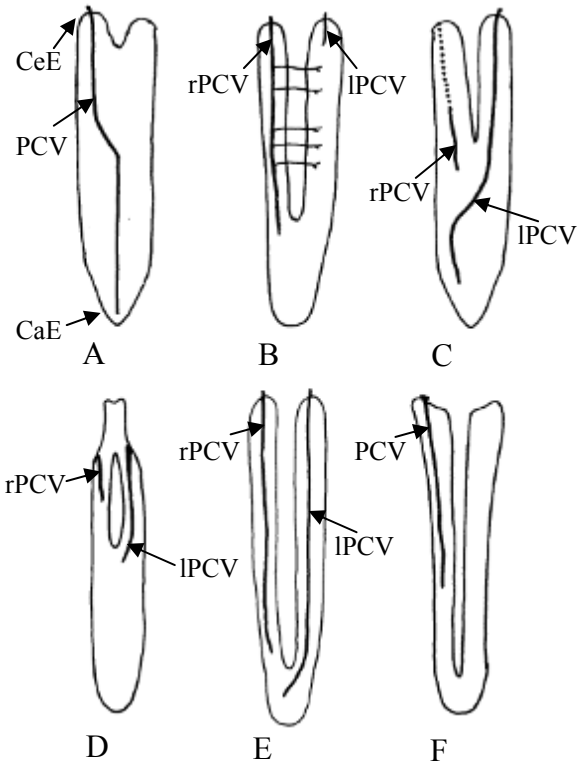


Figure 3.2: Variations in kidney shape and posterior cardinal vein of Anoplarchus purpurescens (A), Anguilla rostrata (B), Acipenser stellatus (C), Polyodon spathula (D), Lepisosteus oculatus (E), Amia calva (F). PCV, posterior cardinal vein; rPCV, right posterior cardinal veins; lPCV, left posterior cardinal vein; CaE, caudal end; CeE, cephalic end. Figure adapted from Mok (1981), Youson et al. (1989).

in the kidney tissue. Towards the cephalic part of the body they unite with the anterior cardinal veins and form the common cardinal vein which also receives blood from the liver and gills ultimately leading to the heart. Major blood vessels are highly specific regarding their anatomical location. Conversely the anatomy of the PCV and indeed the bilateral arrangement of the kidneys are highly variable between species. In cockscomb prickleback, *Anoplarchus purpurescens*, both kidneys are fused together except at the very cephalic end and there is a single PCV related to the right side of kidney which moves to the medial plane in the centre of the animal where it continues caudally as a single vessel (figure 3.2A) (Youson *et al.*, 1989). In the American eel the posterior kidneys are fused together and the left PCV supplies only a small region of the cephalic part of ipsilateral kidney while the right PCV supplies the rest of the kidney (figure 3.2B); in the starry sturgeon, *Acipenser stellatus*, the kidneys are fused at the posterior end and the right PCV supplies the anterior half of the right kidney while the left PCV

crosses the right side in the middle of the kidneys and supplies the posterior region of both kidneys (figure 3.2C); in the Mississippi paddlefish both kidneys are completely fused along their entire length but the anterior region is much smaller than the rest of the kidney and the left PCV supplies the anterior half of the kidney while the right PCV drains blood from only the cephalic end of the ipsilateral kidney (figure 3.2D); in the spotted gar, *Lepisosteus oculatus*, the kidneys are fused only at the posterior end with fully developed right and left PCVs supplying the ipsilateral kidneys from the anterior to the posterior (figure 3.2E). Finally in the bowfin, the renal architecture and the PCV are similar to that seen in the spotted gar with the exception that there is only a single PCV running halfway down the right kidney (figure 3.2F) (Mok, 1981).

As explained earlier, the location of chromaffin tissue varies according to species. Assuming the teleost fish represent the most advanced evolutionary group, chromaffin tissue is present in the head kidney region and is closely associated with major vasculature (Wendelaar-Bonga, 1997). However, in more ancient species chromaffin tissue location is more diverse. In elasmobranchs, chromaffin tissue is related to the paravertebral ganglionic cells (Abrahamsson, 1979), in dipnoans chromaffin tissue is present in intercostal vessels, the heart and cardinal vein (Abrahamsson *et al.*, 1979a, Abrahamsson *et al.*, 1979b, Axelsson *et al.*, 1989, Holmes, 1950) and in agnathans, it is present in the heart (Euler and Fange, 1961, Stabrovskii, 1967). There is little knowledge about the location of chromaffin in sturgeons. In Baltic sturgeon, *Acipenser sturio*, it is related to the walls of PCVs (Giacomini, 1904) and in beluga sturgeon it has been identified in both the PCV and the celiac-mesenteric artery (Balashov *et al.*, 1981, Gallo *et al.*, 2004).

In those vertebrates thus far examined it is understood that chromaffin cells originate from the neuroectodermal layer which are derived from neural crest cells and migrate to their respective areas during development (An *et al.*, 2002, Langley and Grant, 1999). Given all ganglionic cells originate from neural crest cells (Knouff, 1927, Lamers *et al.*, 1981), embryologically, chromaffin tissue represents a specialized ganglionic cell and is typically innervated by preganglionic nerve fibers. However, the timing of the appearance of chromaffin cells during fish development is species dependent. In the

European eel, *Anguilla anguilla*, chromaffin cells were not seen until the elver stage and were present either as single cells or a small group of cells on the dorsal aspect of the vein (Giacomini, 1908). The appearance of catecholamines has been examined in both the embryo and larvae of rainbow trout using biochemical analysis and the earliest stage where catecholamines were detected was the late embryonic period with a significant increase in the concentration of EPI detected at hatch (Meyer and Sauerbier, 1977). Conversely chromaffin tissue in the spotted snakehead, *Channa punctatus*, (Belsare, 1973) and European seabass, *Dicentrarchus labrax*, (Abelli *et al.*, 1993) was not detected until one month after hatching.

Despite the variations in location and timing of appearance, ultra-structure of chromaffin cells share many common characteristics, with some minor differences dependent on whether EPI or NE is the principle secretagogue. In most species they are identified as two separate cells, one being NE and the other EPI secreting. Both cells contain membrane bound small vesicles filled with their respective catecholamine. Vesicles of a NE secreting cell are more or less round in shape with homogenous and dark electron dense granules. Conversely EPI secretory vesicles have variable electron density and are sometimes seen as completely electron lucent. They are round to oval in shape and the contents may show a fine granular appearance (Coupland, 1989). That said, morphological characteristics of these vesicles are highly dependent on the method of fixation. Coupland (1965) studied the chromaffin vesicles with different fixation techniques in the rat adrenal medulla. Using only 1% buffered osmium tetroxide, both vesicles were indistinguishable from each other and were round to be oval in shape with symmetrically arranged electron dense material. However, prior fixation with glutaraldehyde results in a more electron dense material in vesicles where glutaraldehyde reacts with NE forming a complex which displaces it towards the periphery forming asymmetrical and strongly electron dense material (Coupland and Hopwood, 1966). While this explanation represents an artifactual appearance of NE vesicles it is nonetheless a useful tool to differentiate vesicles.

The kidney along with other organs plays a key role for osmoregulation in fish for maintaining ion and water balance (Evans *et al.*, 2005). The structural features of the

functional unit of the kidney, the nephron, depend on the aquatic lifestyle of the fish. Fresh water fishes need to conserve solutes and excrete excess water, and the nephron has a well developed glomerulus, proximal (PT) and distal (DT) tubules and collecting duct (CD) (Edwards, 1928, Majumdar and Drummond, 1999, Nishimura and Fan, 2003, Sakai, 1985). In marine fishes the glomerulus may not as well developed or in some cases absent which is indicative of the need to conserve water (Bulger, 1965, Bulger and Trump, 1968, Edwards, 1928, Ericsson and Olsen, 1970, Olsen and Ericsson, 1968, Ottosen, 1978). In contrast to the number of studies on fresh and marine teleost fishes there are few studies that have described the ultra-structure of sturgeon kidneys (Krayushkina *et al.*, 2001, Krayushkina *et al.*, 1996a, Krayushkina *et al.*, 1996b) and even fewer that have examined the kidneys during development (Abadi *et al.*, 2011).

The original aim of this chapter was to explore the influence of the environment on the development of chromaffin tissue in lake sturgeon larvae. However, for reasons that will be explained this was not possible and so this research examined renal morphology of developing pro-larval and larval lake sturgeon raised at different temperatures and a preliminary examination of chromaffin-like cells in larval lake sturgeon.

3.2 – ***Materials and Methods***

Eggs and milt were obtained from 5 females (31.3 ± 7.28 kg) and 17 males (14.7 ± 1.8 kg) in spring 2009. All fish were captured by gill net at Pointe Du Bois on the Winnipeg River. Gametes from both sexes were thoroughly mixed to broaden the genetic diversity prior to fertilization. Approximately 700 eggs, determined by volume, were measured and placed in a Petri dish. A maximum of three drops of milt were added to each dish before being gentle mixing. Following a 1.5 minute incubation period, 15ml of river water at 10°C was added to promote even distribution of milt and adherence of eggs to the substrate. Samples were gently shaken for 1.5 minutes before the contents of each Petri dish were evenly scattered within experimental tanks. The eggs were then carefully spread across the treatment tanks and allowed to develop in one of 3 different

temperature treatments of 9, 12 and 15°C. Developing oocytes, pro-larvae and larvae were collected throughout development. Following hatch all pro-larvae within a given treatment were moved to a smaller holding tank to allow pro-larvae to burrow in the gravel until emergence when the yolk sac was fully absorbed. Following emergence larvae were fed a diet of *Artemia* sp. *ad libitum* twice daily for a maximum of 5 days at which point sample collection ceased.

To test for the appearance and location of chromaffin tissue, developing pro-larvae were divided into three groups. In the first group, beginning on the first day post fertilization (dpf) 3 individual specimens were collected and fixed at 4°C in a solution of 4% paraformaldehyde (PF) in 0.1M sodium caccodylate buffer (pH 7.4) for 48 hours. In the second group, beginning on the first day post hatch (dph), 3 individual pro-larvae were collected and fixed at room temperature in a dichromate fixative containing 10 volumes of 5% potassium dichromate and 1 volume of 5% potassium chromate (DF) (pH 5.5 to 5.7) for 24 hours. After fixation pro-larvae were kept overnight under running water to wash the excess chromate and dichromate solution from each sample. Following fixation and washing, pro-larvae from both sets were dehydrated in a graded alcohol series, cleared using Slide Bright® as a xylene substitute (Jones Scientific Products, Inc., Kitchener, ON, Canada), and infiltrated and embedded in a tissue prep (Fisher Scientific, Fair lawn, NJ, USA) a paraffin embedding medium for subsequent sectioning. Samples were allowed one change for 20 minutes in each concentrations of 70, 80 and 95% ethyl alcohol (EA) while three changes in 100% EA for 20 minutes and Slide Bright for 30 minutes. They were infiltrated at 60°C under vacuum with two changes of liquid paraffin for one hour each. Slides were prepared from paraffin embedded larvae by sectioning 5-7μm thin sections using a Jung rotary microtome in transverse, lateral and coronal planes. Slides prepared from PF fixation were stained with hematoxylin and eosin (H&E) (Presnell, *et al.*, 1997) in Coplin jars using the following protocol;

1. Three changes in Slide Bright for 2 - 3 minutes each
2. Three changes in 100% EA for 30 seconds each
3. One change in each of 95, 80 & 70% EA for 30 seconds
4. Kept under gently running water for 2 - 3 minutes
5. One change in Modified Harris Hematoxylin stain (Fisher Scientific, Fair lawn, NJ, USA) for 3 - 5 minutes

6. 5 to 10 dips in acid alcohol solution* (destaining excess hematoxylin)
7. Kept under gently running water for a few seconds
8. One change in Scott's solution** for 3 minutes
9. Kept under gently running water for 2 minutes
10. One change in 70% EA for 10 seconds
11. One change in Eosin Y stain*** for 1 - 3 minutes
12. One change in each of 70, 80 & 95% EA for 10 seconds
13. Three changes in 100% EA for more than 1 minute each
14. Three changes in Slide Bright for 2 - 3 minutes each
15. One change in 100% xylene for more than 2 minutes

* 0.0025% HCl in 70% EA
** 0.068M lithium carbonate (Sigma-Aldrich, Inc., St. Louis, USA) in deionized water
*** 500mg of Eosin Y (Sigma-Aldrich, Inc., St. Louis, USA) (or 50ml of 1% aqueous solution) in 150ml of 95% EA and 1ml glacial acetic acid

Slides prepared from DF fixation were further stained with hematoxylin in the same manner as described above but omitting steps 10 & 11. All stained slides were dehydrated, cleared and mounted in permount (Fisher scientific, Fair lawn, NJ, USA) with 24x50-1 cover slips. Fixation of pro-larvae in PF allowed for anatomical examination. DF was used to identify chromaffin tissue as potassium dichromate is known to react with the amino group of catecholamines and imparts a yellow to dark brown color in the EPI and NE containing cells (Hillarp and Hokfelt, 1955, Lever *et al.*, 1976, Wood *et al.*, 1971). Eggs hatched at 6-8, 9-11 and 15-17 dpf for the 15°, 12° and 9°C treatment groups respectively and were considered pro-larvae at this stage with the yolk sac attached. Fish began feeding 9-11, 16-18 and 35-37 dph in the 15, 12 and 9°C treatment groups respectively and were considered larvae at this stage as all the yolk sac had been absorbed. For fixing in PF and DF, samples were collected daily until 12, 21 and 18 dph for the 15°C, 12°C and 9°C treatment groups respectively. To study final development of pro-larvae samples were collected of 27 and 41 dph for the 12°C and 9°C treatment groups respectively. As a positive control head kidneys of goldfish, *Carassius auratus*, were fixed with PF and DF in a similar fashion. After embedding in paraffin wax, 10 slides were prepared from each condition. Two slides prepared from the DF solution were further stained with hematoxylin and two slides from tissue fixed in PF only were stained with H&E. These slides were mounted in a similar fashion as above. All slides were viewed using a Zeiss Universal microscope for bright field light microscopy (Zeiss, Germany) using 4, 6.3, 10, 20, 40 and 60X Nikon objective lenses

(Tokyo, Japan). Areas of interest on the slide were captured through a Sony DXC-390P, 3CCD color video camera (Tokyo, Japan), a Matnox frame grabber in a PC and images processed via Northern Eclipse software, v. 6.0 (Empix imaging, Inc., Mississauga, ON, Canada). Goldfish head kidney was used as a positive control as it is well recognized that chromaffin tissue is found in abundance in this region of the kidney in teleost fish (Wendelaar-Bonga, 1997). Pro-larvae were examined for presence or absence of staining indicative of chromaffin tissue alongside the appearance of the renal tissue.

A third set of samples, containing 3 larvae, were fixed in modified Karnovsky's fixative (1% paraformaldehyade and 2.5% glutaraldehyde) in 0.1M cacodylate buffer (pH 7.2) for 48 hours and post-fixed in 2% osmium tetroxide in 0.1M cacodylate buffer for 3 hours. After dehydrating in a graded alcohol series they were stored in 100% EA at -20°C for 24 hours in preparation of the following phase. All fixation and dehydration steps were carried out at 4°C. Individual samples were taken and embedded in an Epon araldite embedding mixture (Mollenhauer, 1964) as follows;

1. Samples were allowed to reach room temperature
2. Three changes in 100% EA for 2 minutes each
3. One change in 1:1 mixture of 100% EA and acetone for 20 minutes
4. Two changes in 100% acetone for 20 minutes each
5. Samples then left in 1:1 mixture of acetone and catalyzed epon (2 – 3% DMP-30 in epon araldite) (Electron Microscopy Sciences, Hatfield, PA, USA) overnight. Subsequently the acetone was allowed to evaporate in fume hood thereby increasing the concentration of infiltration solution.
6. After 24 hours the samples were transferred to freshly catalyzed epon for two changes of 24 hours each
7. Samples were then placed in embedding molds with freshly prepared catalyzed epon and allowed to polymerize at 60°C for at least 24 hours

All these steps were carried out at room temperature. Semi-thin sections of 1.5µm thickness were cut on a glass knife using a Richert-Jung Ultracut Microtome (A-1170 Wein, Austria) and stained with 1% Toluidine blue in 1% Borax by adding a few drops to heated sections fixed to glass slides for 10 – 20 seconds. Sections were then mounted in immersion oil with 22x22-1.5 cover slips and images were taken as described earlier. For transmission electron microscope (TEM) silver and gold ultra-thin sections were cut on a glass knife using the same Ultracut microtome and mounted on a 200 mesh copper EM

grids. Unstained grids were examined using a Hitachi H-7000 STEM in TEM mode at various magnifications ranging from 1,000X to 80,000X using a digital CCD camera (Advanced Microscopy Techniques, Woburn, MA, USA) and images were captured using a Image Capturing Engine Software v. 601.384 (Advanced Microscopy Techniques, Woburn, MA, USA).

*3.3 – **Results***

3.3a Positive Control:

Chromaffin tissue in the positive control of the goldfish head kidney fixed with DF was recognized as having a light to dark brown color under the light microscope. The chromaffin tissue of samples stained with H&E reacted with hematoxylin only and imparted a blue color due to their basophilic nature (see section 1.1a). This tissue was seen as a cluster of cells dispersed in the head kidney of the goldfish (figure 3.3).

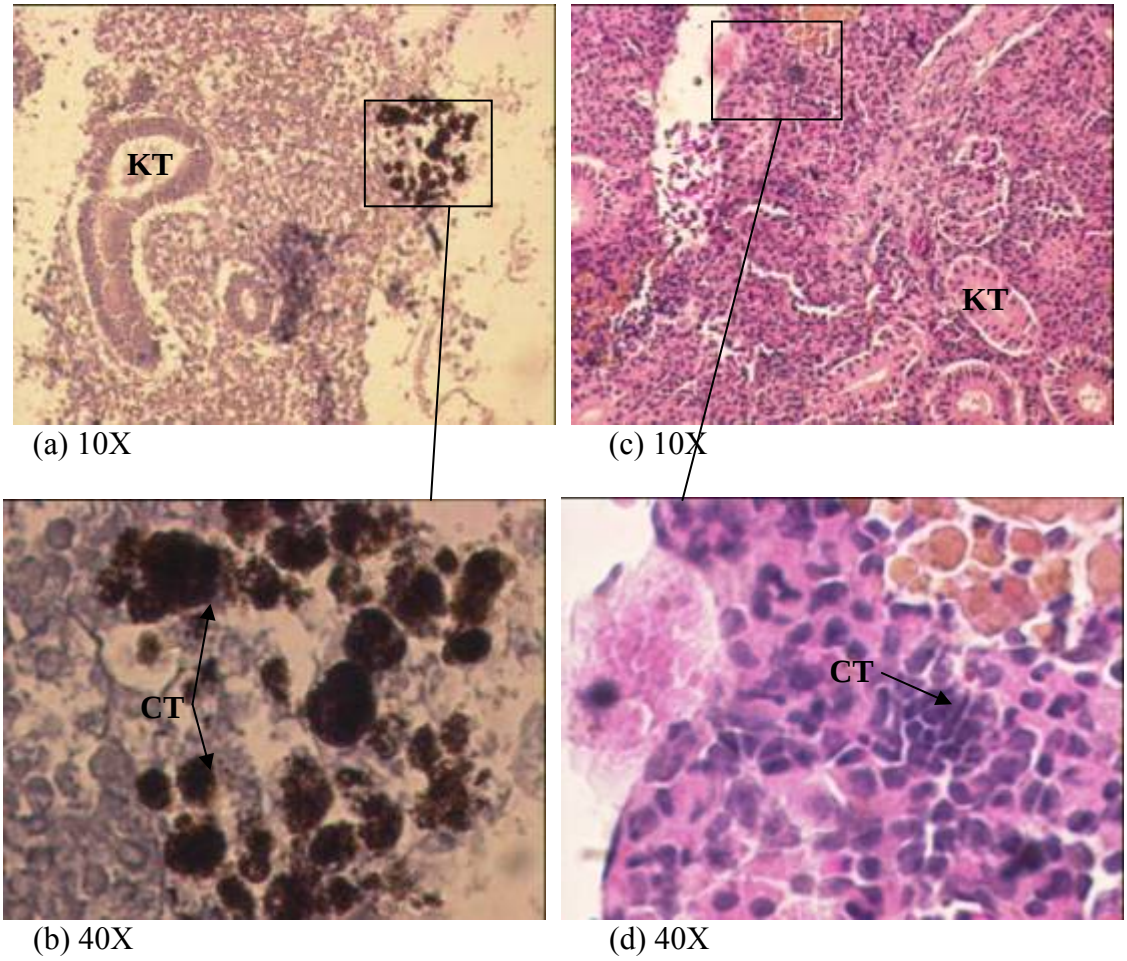


Figure 3.3: (a & b) Dichromate and (c & d) buffered formalin fixed head kidney of the gold fish stained with haematoxylin and haematoxylin and eosin respectively showing chromaffin tissue (CT) and kidney tubule (KT).

3.3b Light Microscopy:

Chromaffin tissue appearance in pro-larvae and larval fish:

Regrettably, fixation and staining techniques used in the present study did not allow for the identification of chromaffin tissue using light microscopy in any of the treatment groups. Figures 3.8, 3.13 and 3.15 show transverse sections of sturgeon pro-larvae fixed in modified Karnovsky's fixative, DF and PF respectively. Dark brown to black pigments in melanocytes were consistently present in all three fixed samples at a similar location, i.e., below the skin, on the outer surface of either the alimentary canal or yolk sac and within renal tissue (figure 3.14c). Chromaffin tissue could impart a dark brown color following fixation in the DF, however, the samples fixed in PF suggested that this was not the case. This was further supported by the transverse view of pro-larvae fixed in modified Karnovsky's fixative. Reasons for the apparent lack of chromafin tissue using these techniques are unclear but may be due to the pH of the DF used to fix the larvae, sensitivity of the staining technique used and/or immaturity of the chromaffin cells. Thus the following focuses on the development of the renal tissue in the different temperature treatments.

Kidney development in pro-larvae and larval fish:

Figures 3.13 and 3.15 show transverse views of pro-larvae fixed with DF and PF respectively. Renal tissue was evident in pro-larvae of all treatments and the kidney could be divided into 3 distinct regions, anterior, middle and posterior. Morphologically, the anterior region was found to be the shortest and thickest and was located dorsally and laterally to the alimentary canal or yolk sac and medially and ventrally to the developing skeletal muscle (figures 3.6, 3.10, 3.13a & 3.15a-e). The middle portion of the kidney runs dorsally and spreads medially and represents the widest portion in regard to both a transverse and ventral dorsal aspect (figure 3.10, 3.13c-d, 3.14 & 3.15f-p). During the earlier stages of development the middle kidney of the pro-larvae appears to be present only on the lateral aspects of the body (figures 3.9a-b) and as development progresses the

kidney tubules expand medially so that at the larval stages the kidneys of both sides are separated at the midline by a small space (figure 3.10a-b & 3.15f-p). The posterior kidney is the longest region of the kidney and runs dorsal to the anus or yolk sac and ventral to the neural tube (figure 3.10c-d, 3.12b & 3.13e-f).

A typical pro-larval kidney appeared to consist of four distinct regions including, the pronephric tubule (PnT), PT, DT and CD. Glomeruli were typically absent at the pro-larval stage but started to appear at larval stage (figure 3.12b). Identification and location of these different regions was based on the histological differences observed in the larvae fixed in PF (figure 3.14 & 3.15).

The epithelial cells lining the presumed PnT had a distinct brush border but they were less columnar in appearance than the epithelial cells lining the PT. Furthermore this section of the kidney tubule appeared less convoluted than the PT and was found throughout the kidney with the exception of the cephalic area of the anterior kidney. In some sections there was a loss of brush boarder while in others there was vacuolization observed in the cytoplasm of the epithelial cells. Structurally the main feature that differentiated the PnT and PT from other regions was the presence of a brush border. In addition the epithelial cells lining the PT were columnar in appearance as opposed to the cuboidal nature of the epithelial cells lining the DT and CD. The PT is generally considered the most convoluted region of the kidney tubule and can be sub-divided into anterior (APT) and posterior (PPT) proximal tubules. The main feature by which APT and PPT were differentiated was the size of the lumen, that is the APT lumen was generally narrower than the PPT. The APT was concentrated toward the medial and dorsal sides of the anterior and middle regions of the kidney and it is likely that the brush border in this region of the renal tubule represents an area of maximum absorptive function. The PPT was present laterally and ventrally to the APT and appeared to have greater convolutions than the PPT in the anterior region, while the APT appeared to have greater convolutions in the middle region of the kidney. Epithelial cells lining the DT did not appear to contain a significant brush border and for the most part were less convoluted and appeared in the lateral and ventral regions of the kidney. The epithelial cells of the DT were cuboidal in shape and had a distinct round shaped nucleus. In lateral

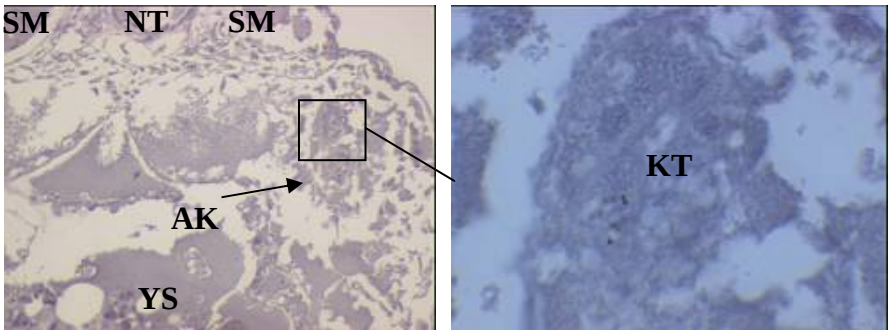
sections the CD was observed on the most ventral side of the kidney running longitudinally from the cephalic to caudal plane of larvae in the middle kidney. The epithelial cells of the CD were more cuboidal in shape compared to the DT, furthermore the nuclei were more rounded and centrally located and the plasma membranes of the epithelial cells in the CD were more clear and pronounced compared with epithelial cells of the DT.

Influence of temperature on developmental rate of the kidney:

In present study there was no difference in the pattern of development between any treatments, however, the different temperature treatments did influence the rate of kidney development. At 11 dph the middle region of the kidney in the 9°C treatment group was less developed and was still only located in the lateral regions (figure 3.5), whereas at a similar time point the kidney in the 15°C treatment group was more developed and fully occupied the dorsal surface of the alimentary canal (figure 3.12a) and the 12°C treatment group represented a medial version of the two temperature extremes at the same time point (figure 3.9a-b). Similarly the anterior and posterior regions of the kidneys in the 15°C treatment group (Figure 3.11 & 3.12b) were thicker and the 9°C treatment group (figure 3.4 & 3.5) were thinner as compared to the group raised at 12°C (figure 3.7).

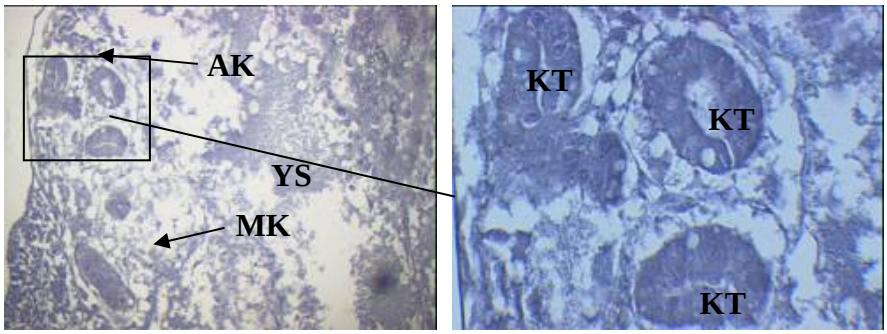
Table 3.1: Summary of light and electron microscopy images. H, hematoxylin; H&E, hematoxylin and eosin; KV, modified Karnovsky's fixative; OT, osmium tetroxide; DF, dichromate fixative; PF, paraformaldehyde; TB, toluidine blue.

Figure #	Temperature (°C)	Age (Day post hatch)	Fixation	Plane	Staining
3.4	9	6	DF	Transverse	H
3.5	9	11	DF	Coronal	H
3.6	9	41	DF	Coronal	H
3.7	12	6	DF	Transverse	H
3.8	12	8	KV, OT	Transverse	TB
3.9	12	11	DF	Coronal	H
3.10	12	27	DF	Coronal	H
3.11	15	6	DF	Transverse	H
3.12	15	11	DF	Coronal	H
3.13	15	12	DF	Transverse	H
3.14	15	10	PF	Lateral	H&E
3.15	15	12	PF	Transverse	H&E
3.16	12	14	KV, OT	-	None
3.17	12	14	KV, OT	-	None
3.18	12	14	KV, OT	-	None
3.19	12	14	KV, OT	-	None



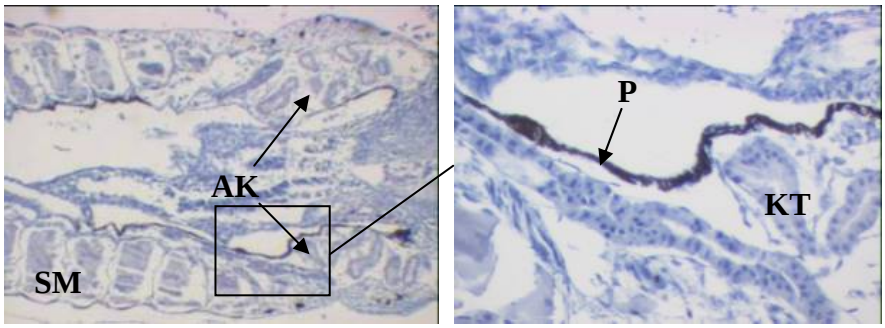
(a) 6.3X (b) 40X

Figure 3.4: 5µm thick transverse section of a 6 dph sturgeon pro-larva raised at 9°C at the level of anterior kidney. The pro-larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AK, anterior kidney; KT, kidney tubule; NT, neural tube; SM, skeletal muscles; YS, yolk sac.



(a) 6.3X (b) 20X

Figure 3.5: 7µm thick coronal section of an 11 dph sturgeon pro-larva raised at 9°C. The section was made toward the dorsal side of the fish showing both anterior and middle kidney. The pro-larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AK, anterior kidney; KT, kidney tubule; MK, middle kidney; YS, yolk sac.



(a) 4X (b) 10X

Figure 3.6: 6 µm thick coronal section of a 41 dph sturgeon larva raised at 9°C. The section was made toward the ventral side of the fish and middle kidney is absent. The larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AK, anterior kidney; KT, kidney tubule; P, pigment; SM, skeletal muscles.

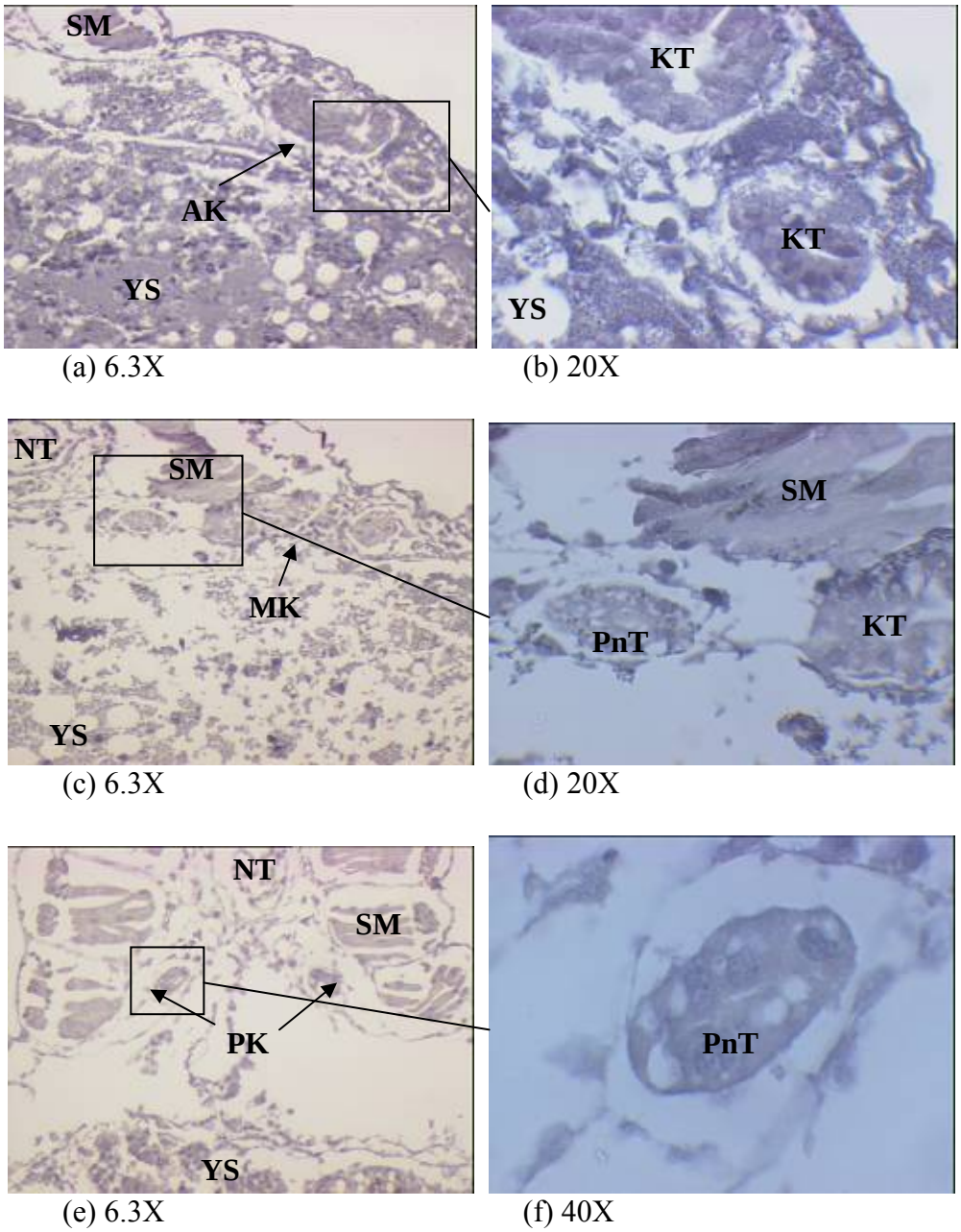


Figure 3.7: 5 µm thick transverse sections of a 6 dph sturgeon pro-larva raised at 12°C at the level of anterior kidney (a & b), middle kidney (c & d) and posterior kidney (e & f).The pro-larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AK, anterior kidney; KT, kidney tubule; MK, middle kidney; NT, neural tube; PK, posterior kidney; PnT, pronephric tubule; SM, skeletal muscles; YS, yolk sac.

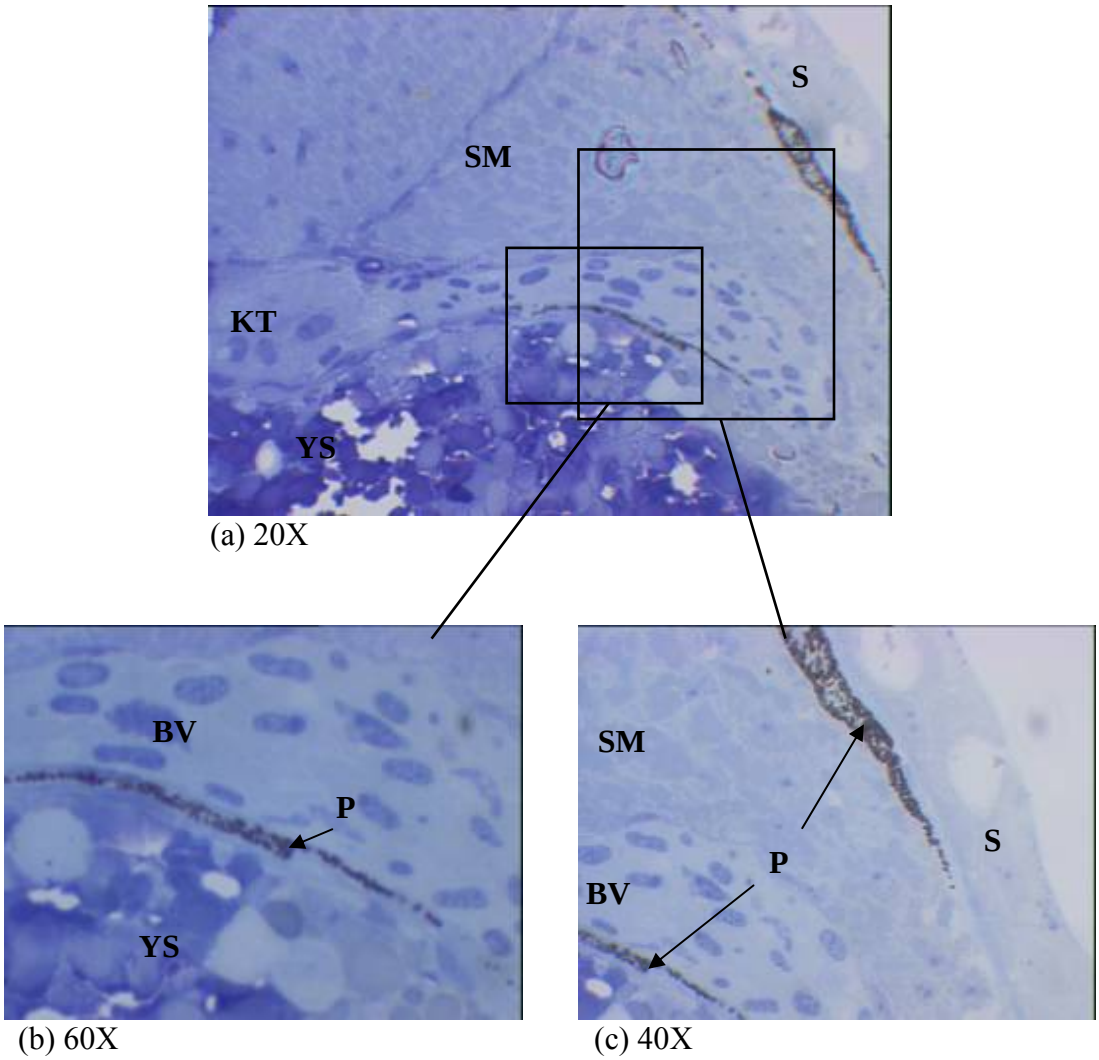
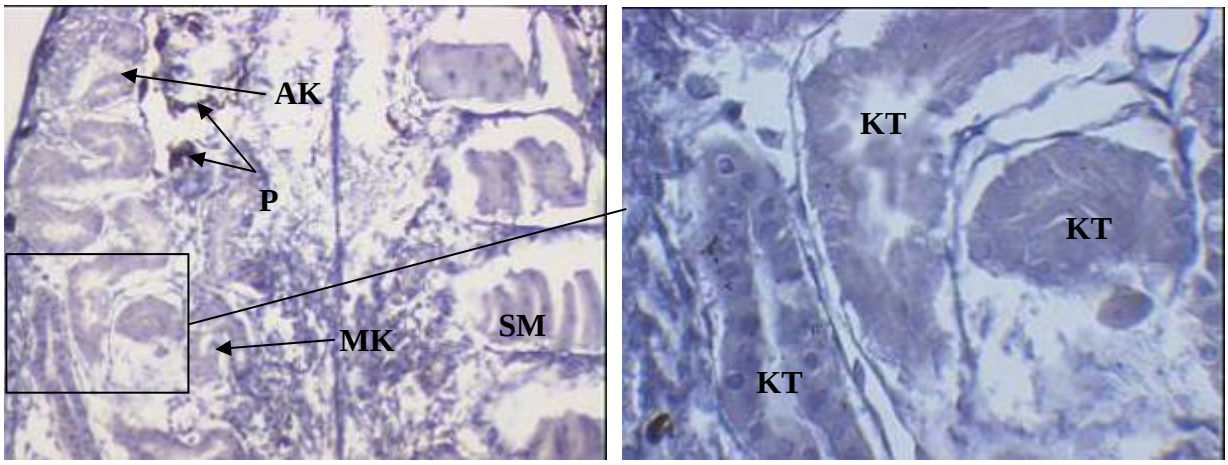
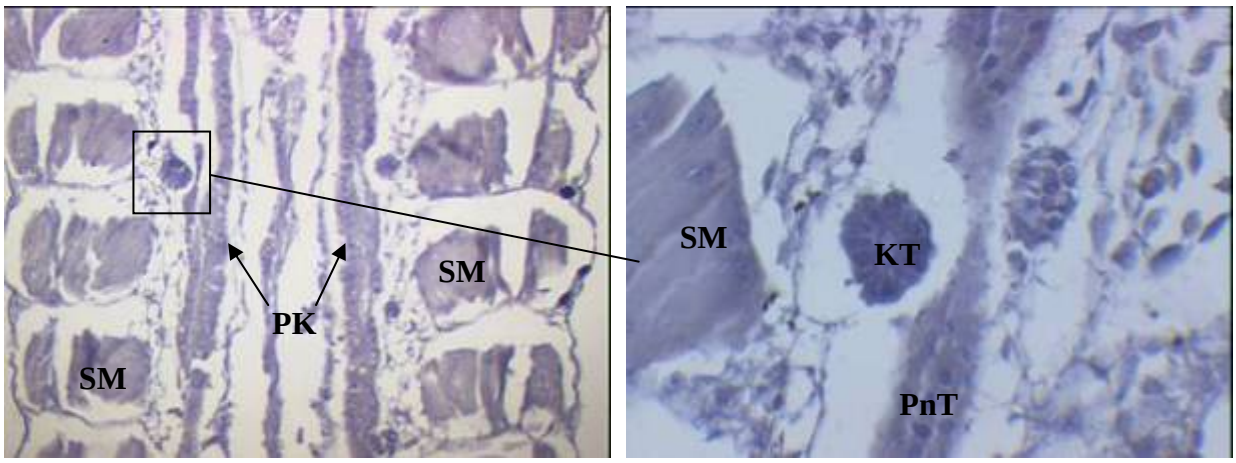


Figure 3.8: 1.5µm thick transverse sections of an 8 dph sturgeon pro-larva raised at 12°C. The pro-larva was fixed in modified Karnovsky's fixative and osmium tetroxide and stained with 1% toluidine blue. BV, blood vessel; KT, kidney tubule; P, pigment; S, skin; SM, skeletal muscle.



(a) 6.3X (b) 20X



(c) 6.3X (d) 20X

Figure 3.9: 7µm thick coronal sections of an 11 dph sturgeon pro-larva raised at 12°C. The pro-larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AK, anterior kidney; KT, kidney tubule; MK, middle kidney; P, pigment; PK, posterior kidney; PnT, pronephric tubule; SM, skeletal muscle; YS, yolk sac.

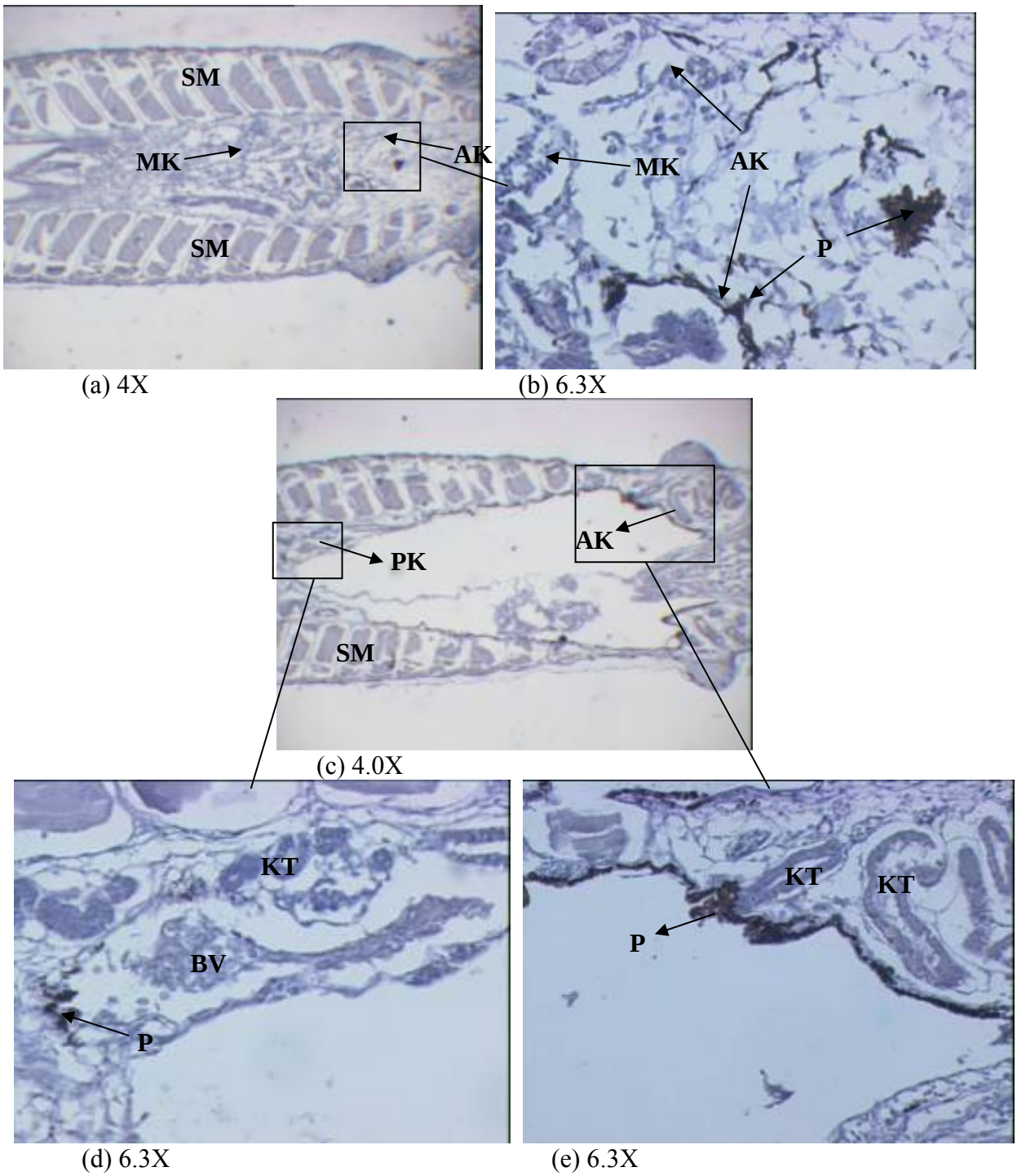
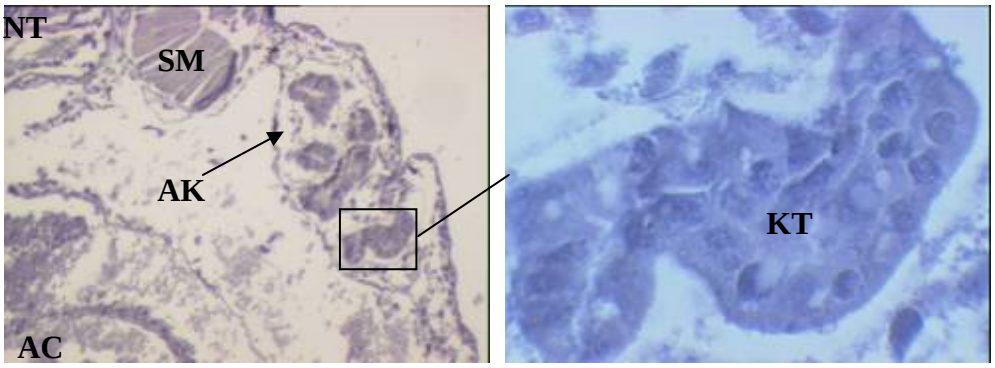
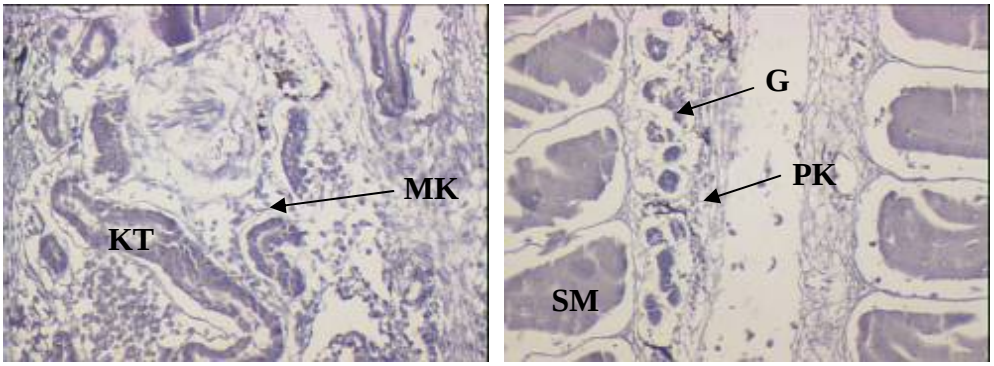


Figure 3.10: 7 µm thick coronal sections of a 27 dph sturgeon larva raised at 12°C. Section (a) & (b) are towards the dorsal side showing anterior and middle kidneys while sections (c) to (e) are more towards the ventral side where middle kidney is absent. The larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AK, anterior kidney; BV, blood vessel; KT kidney tubule; MK, middle kidney; P, pigment; PK, posterior kidney; SM, skeletal muscle.



(a) 6.3X (b) 40X

Figure 3.11: 5µm thick transverse sections of a 6 dph sturgeon pro-larva raised at 15°C at the level of anterior kidney. The pro-larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. AC, alimentary canal; AK, anterior kidney; KT, kidney tubule; NT, neural tube; SM, skeletal muscles.



(a) 6.3X (b) 6.3X

Figure 3.12: 5µm thick coronal sections of a 11 dph sturgeon larva raised at 15°C. Section (a) is through the middle of the fish showing the middle kidney and (b) is the posterior part of abdomen showing the posterior kidney. The larva was fixed in potassium dichromate solution and sections were stained with hematoxylin stain. G, Glomerulous; KT, kidney tubule; MK, middle kidney; PK, posterior kidney; SM, skeletal muscles.

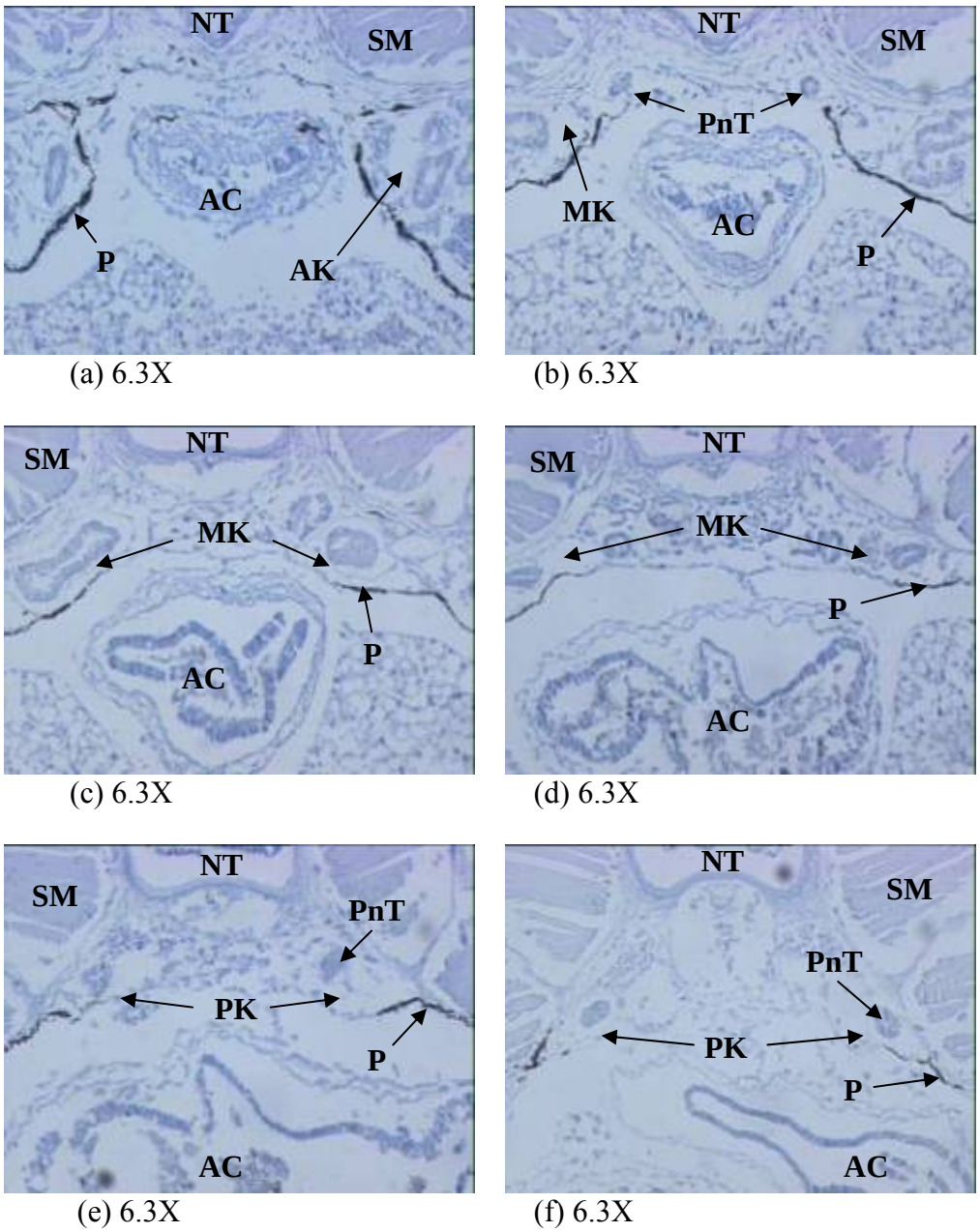


Figure 3.13: 5µm thick transverse views of a 12 dph sturgeon larva raised at 15°C. Section (a) is the cephalic end while subsequent sections represent increasingly caudal views. The larva was fixed in potassium dichromate solution and stained with hematoxylin stain. AC, alimentary canal; AK, anterior kidney; MK, middle kidney; NT, neural tube; P, pigment; PK, posterior kidneys; PnT, pronephric tubule; SM, skeletal muscle.

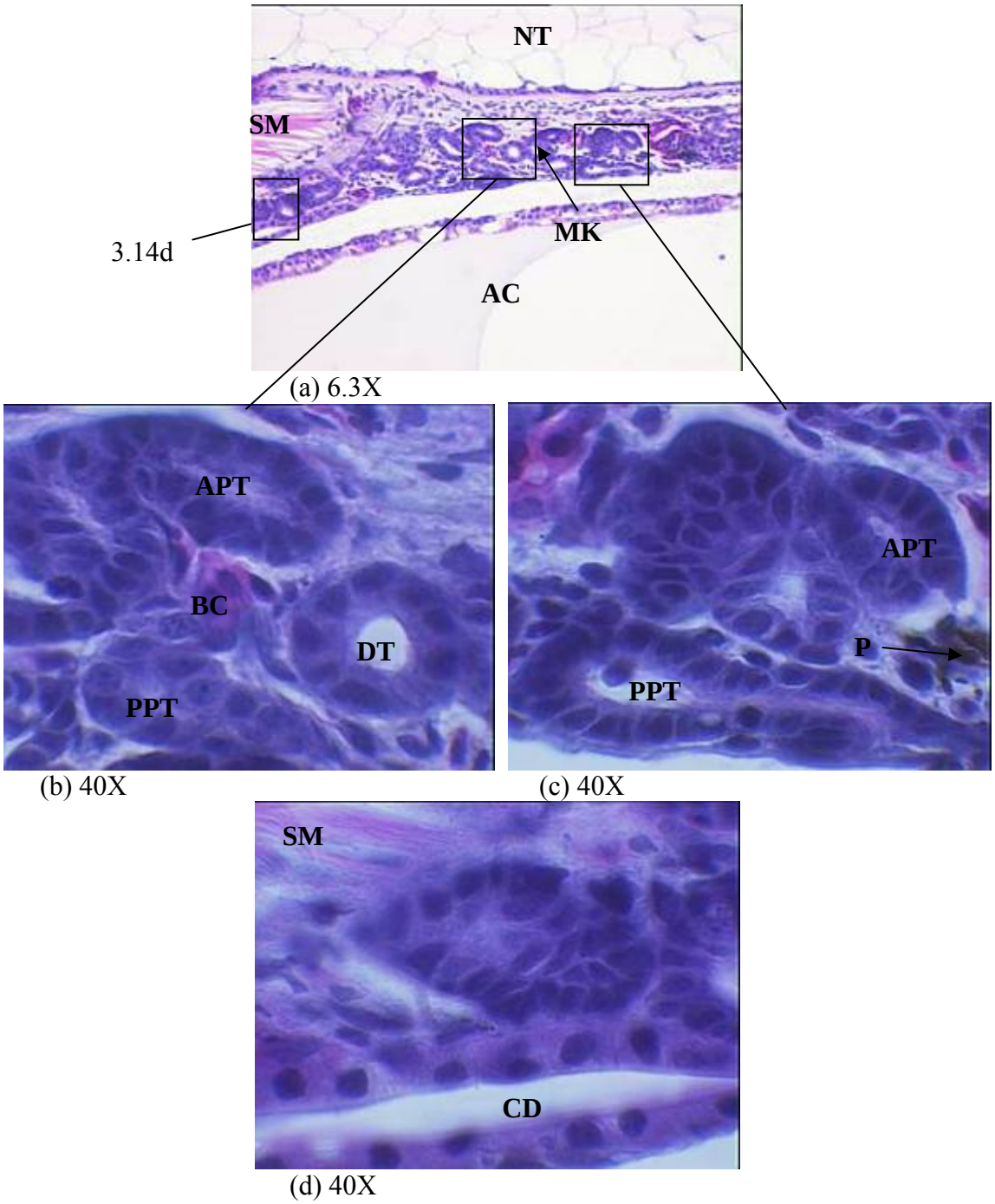
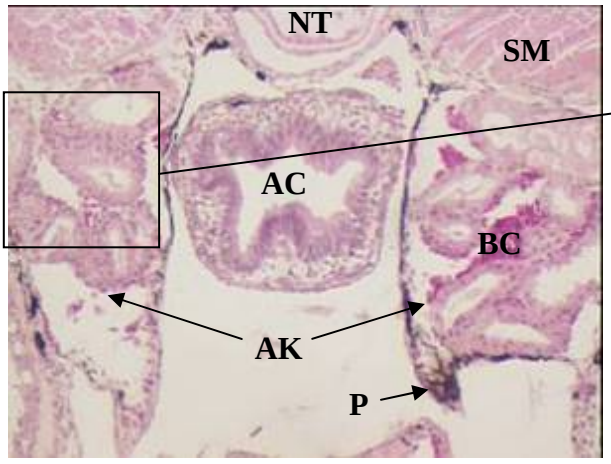
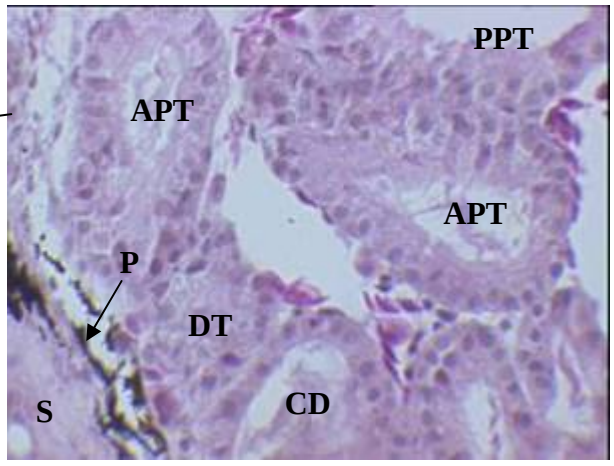


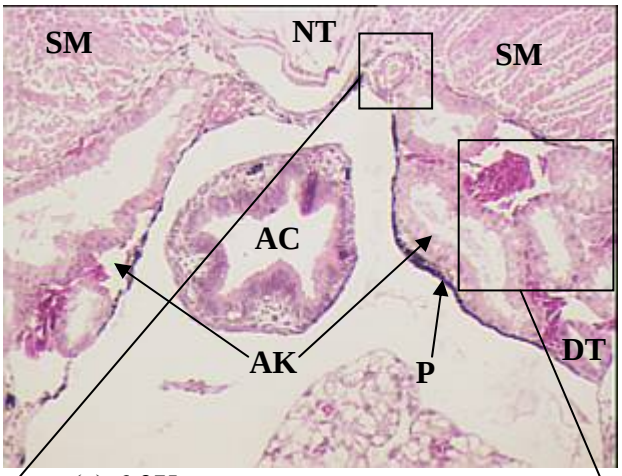
Figure 3.14: 6µm thick lateral section of a 10 dph sturgeon pro-larva raised at 15°C at the level of the middle kidney. The pro-larva was fixed in paraformaldehyde and sections were stained with hematoxylin and eosin. The left side of panel (a) is caudal to the fish while the top of panel (a) is the dorsal surface of the fish. AC, alimentary canal; APT, anterior proximal tubule; BC, blood cells; CD, collecting duct; DT, distal tubule; MK, middle kidney; NT, neural tube; P, pigment; PPT, posterior proximal tubule; SM, skeletal muscles.



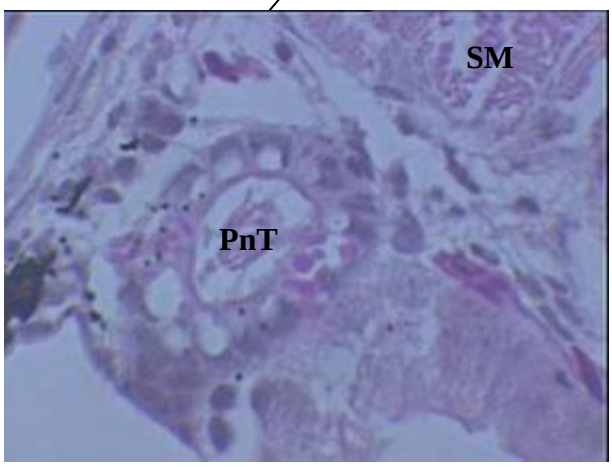
(a) 6.3X



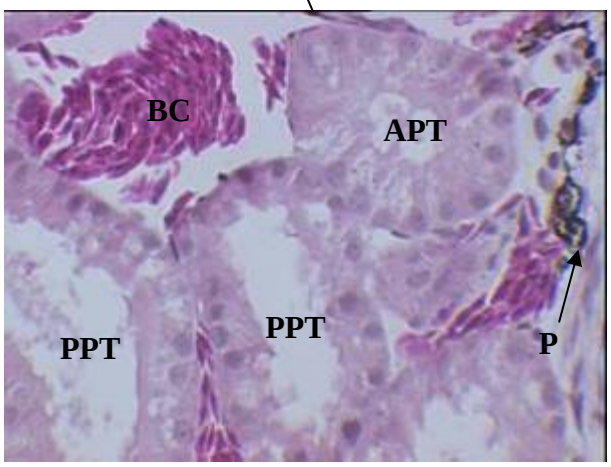
(b) 20X



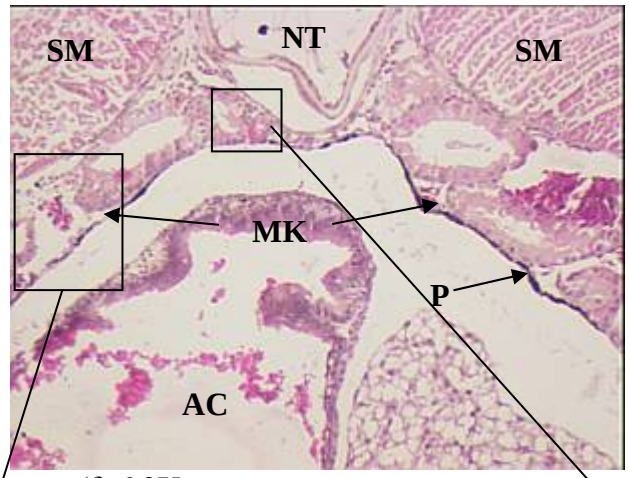
(c) 6.3X



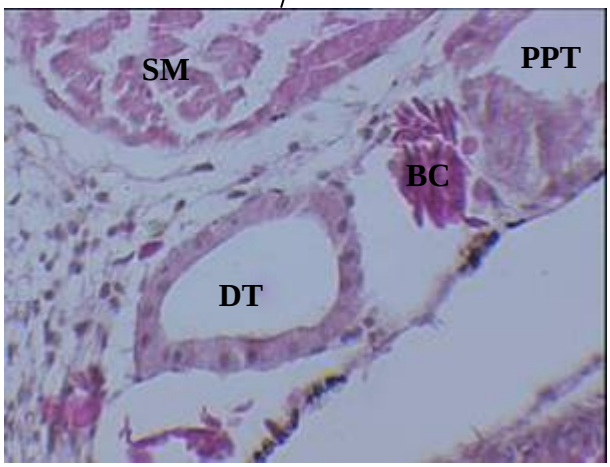
(d) 40X



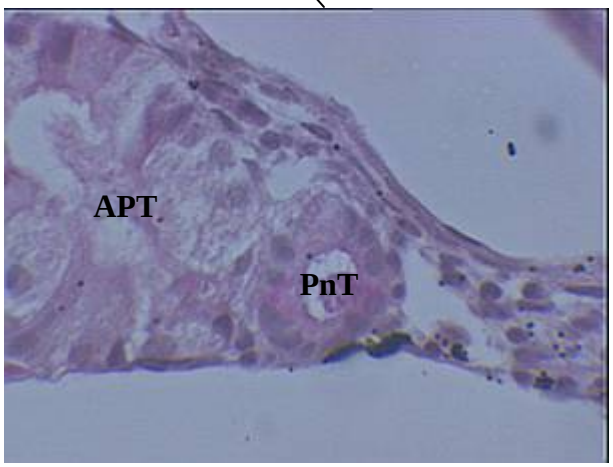
(e) 20X



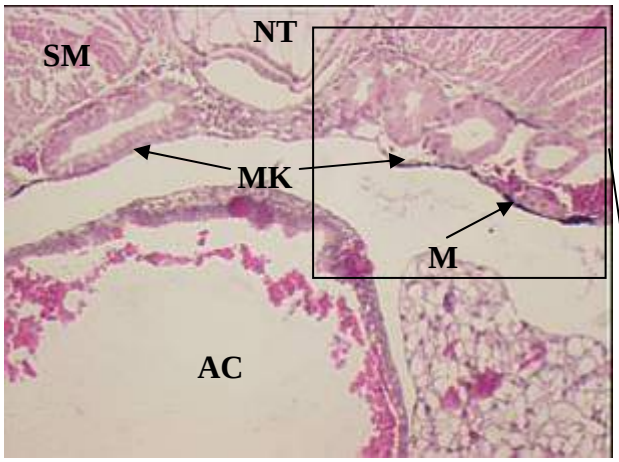
(f) 6.3X



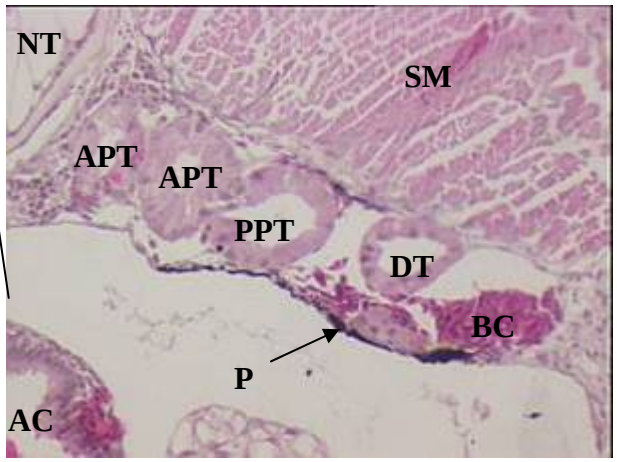
(g) 20X



(h) 20X



(i) 6.3X



(j) 10X

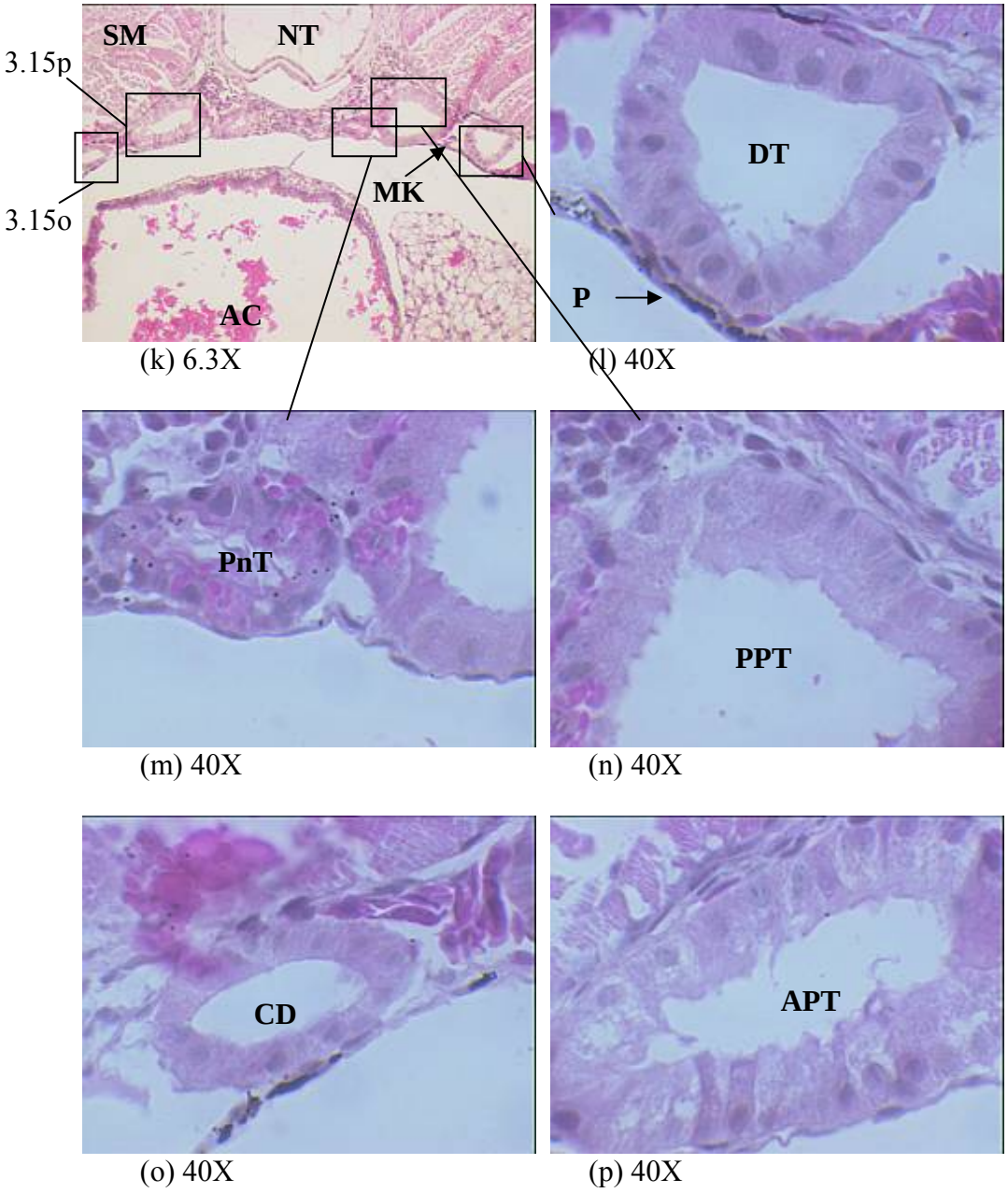


Figure 3.15: 5µm thick transverse views of a 12 dph sturgeon larva raised at 15°C through anterior (a-e) and middle (f-p) kidneys. The larva was fixed in paraformaldehyde and sections were stained with hematoxylin and eosin. Section (a) is the cephalic end while sections (c), (f), (i) and (k) represent increasingly caudal views. Sections l to p represent a higher magnification of the distal tubule, pronephric tubule, posterior proximal tubule, collecting duct and anterior collecting duct respectively. AC, alimentary canal; AK, anterior kidney; APT, anterior proximal tubule; BC, blood cells; DT, distil tubule; MK, middle kidney; P, pigment; PnT, pronephric tubule; PT, neural tube; PPT, posterior proximal tubule; SM, skeletal muscles.

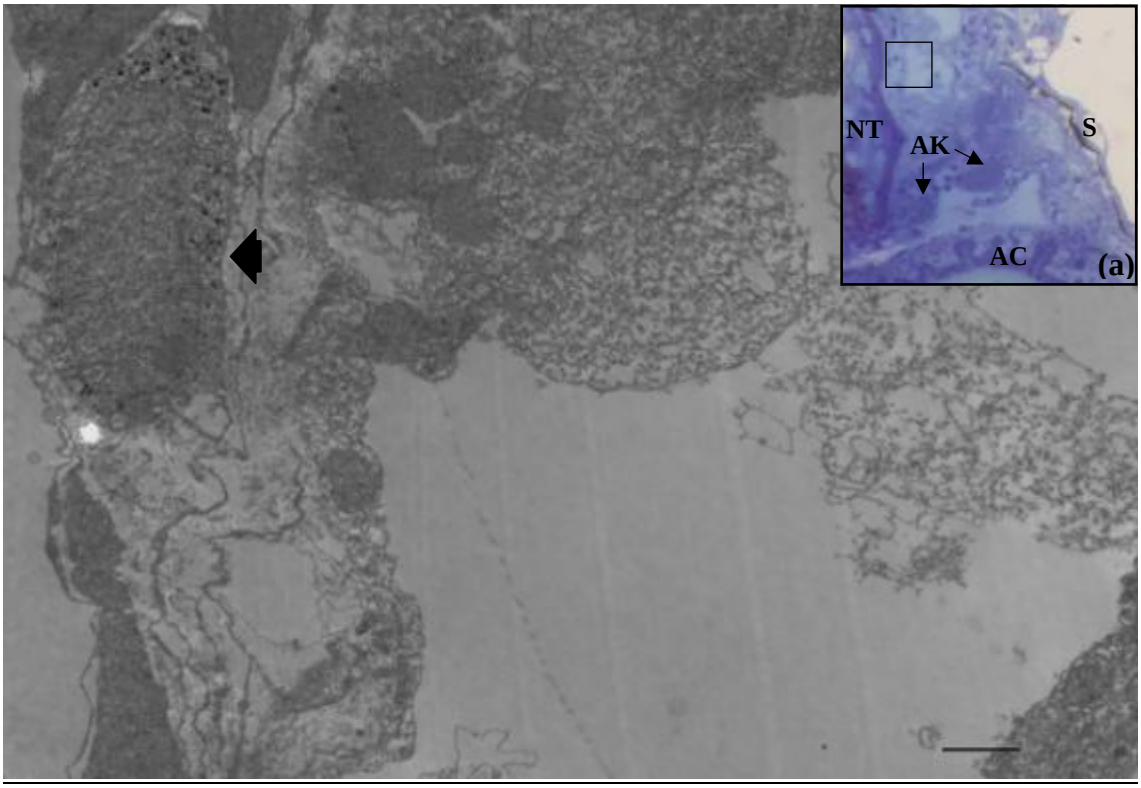
3.3c Electron Microscopy:

Using TEM chromaffin-like cells based on them containing a high concentration of secretory vesicles were identified at different locations. The first was near the neural tube (figure 3.16) and second was in close association with the cardinal vein (Figure 3.17). A third and more surprising location was chromaffin-like vesicles in close association with PT cells of the kidney (figure 3.18 & 3.19).

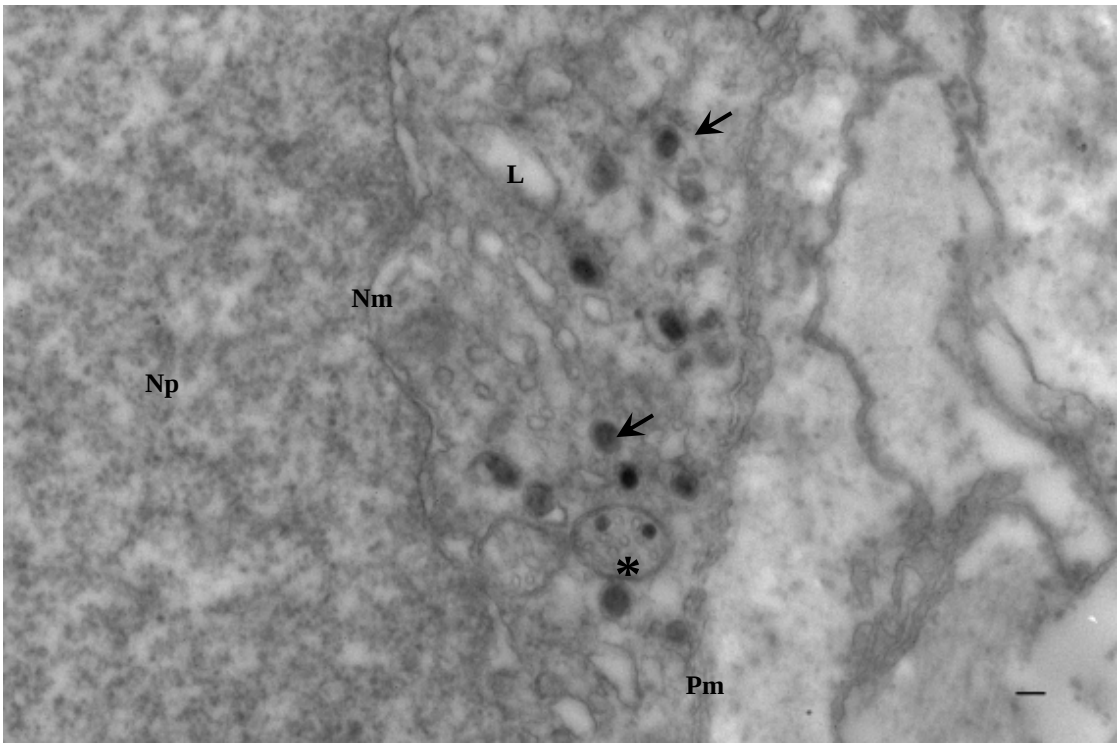
Chromaffin cells related to the cardinal vein were found as single elongated cells containing two types of secretory vesicles; one containing EPI like secretory granules and other NE like secretory granules. EPI vesicles were seen as homogenous and symmetrical, with an average size of approximately 100 nm in diameter. The contents were separated from the vesicular membrane by a thin space. NE like vesicles were more asymmetrical and were relatively larger in size with an average diameter of approximately140nm. Although the vesicles were not counted in the present study relatively fewer NE like vesicles were seen compared to EPI like vesicles. The cytoplasm of the chromaffin-like cells identified in the present study was rich in smooth endoplasmic reticulum, lipid droplets and mitochondria, indicating a high energy demand of these cell types.

Chromaffin-like vesicles associated with the PT of the kidney were distinctly different from those identified in association with the cardinal vein. The vesicles were highly abundant and packed in a membrane bound sac-like structure that was aligned with membrane invaginations of the PT cells. Furthermore, only a single type of secretory vesicle was observed. The contents were not separated from the membrane, the diameter was smaller (approximately 60nm) and there was an increased variability in electron density than the observed NE and EPI like vesicles that were related to the blood vessels.

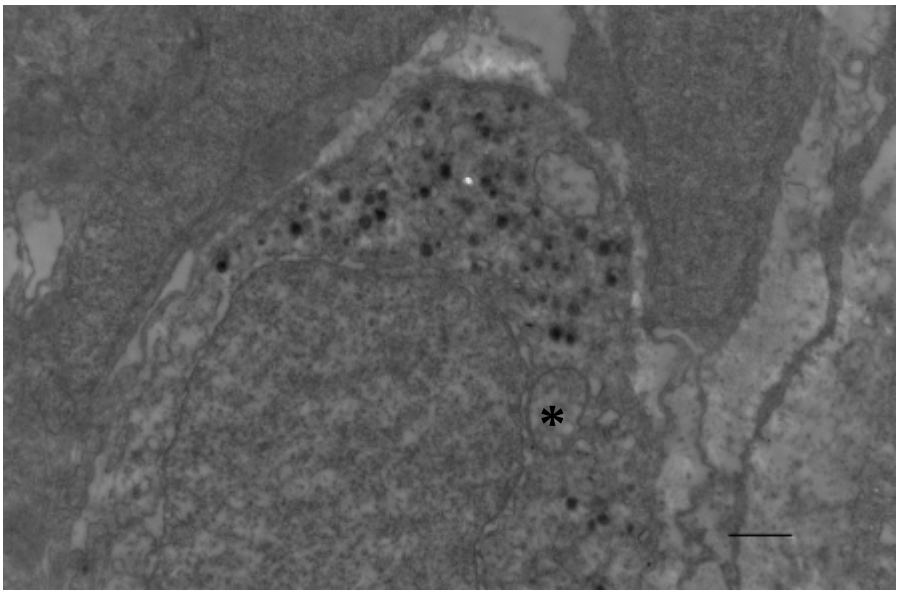
Electron Micrographs:



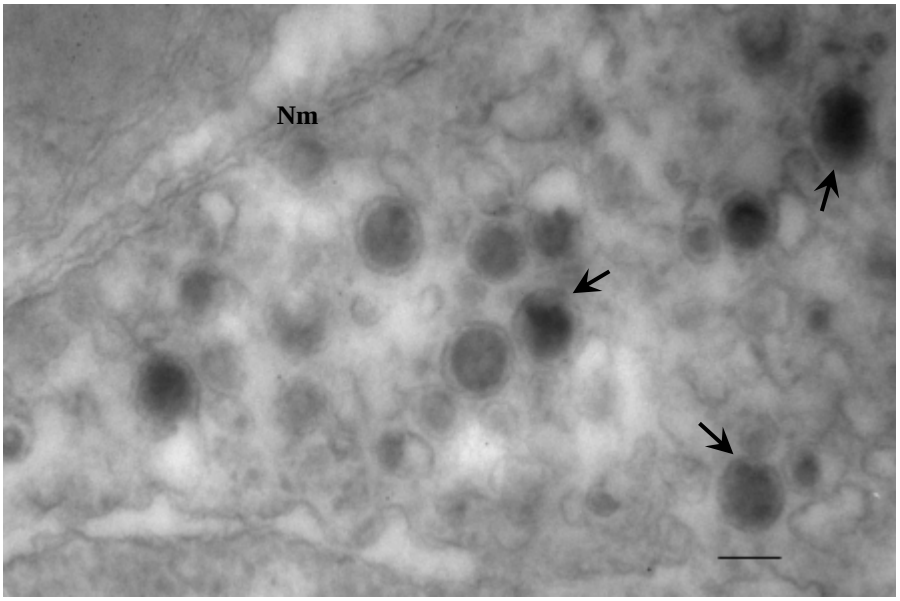
(b) 2000X. Bar = 2µm.



(c) 15,000X. Bar = 100nm.

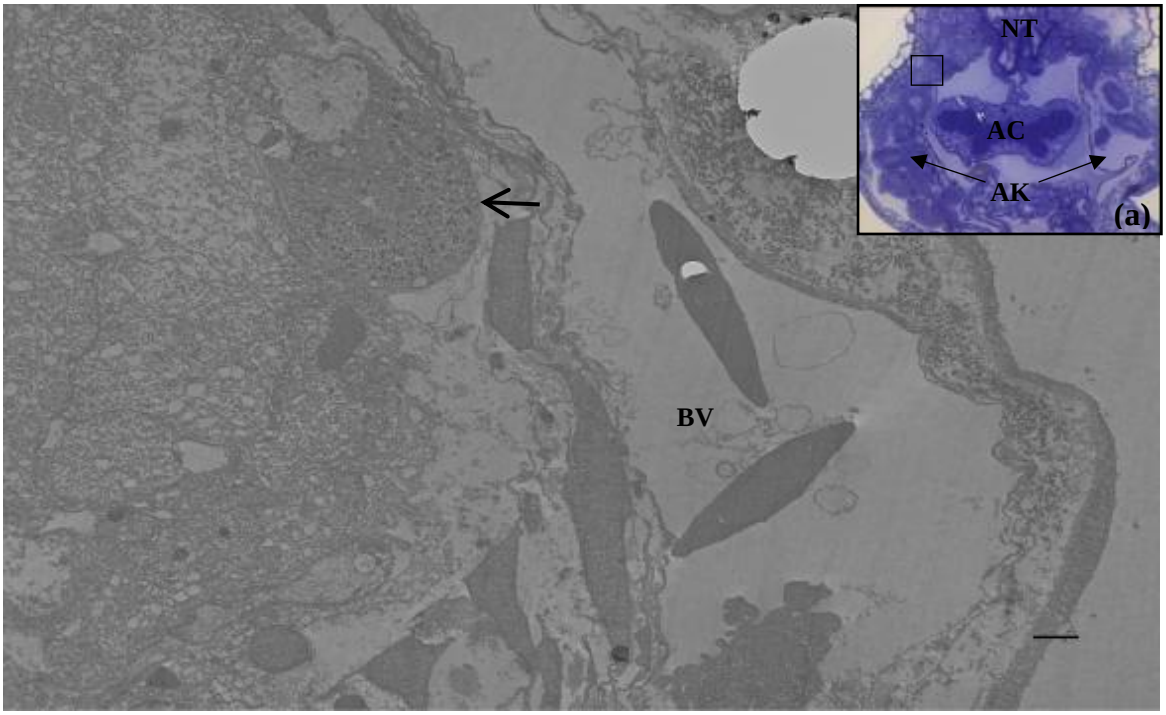


(d) 8000X, Bar = 500nm.

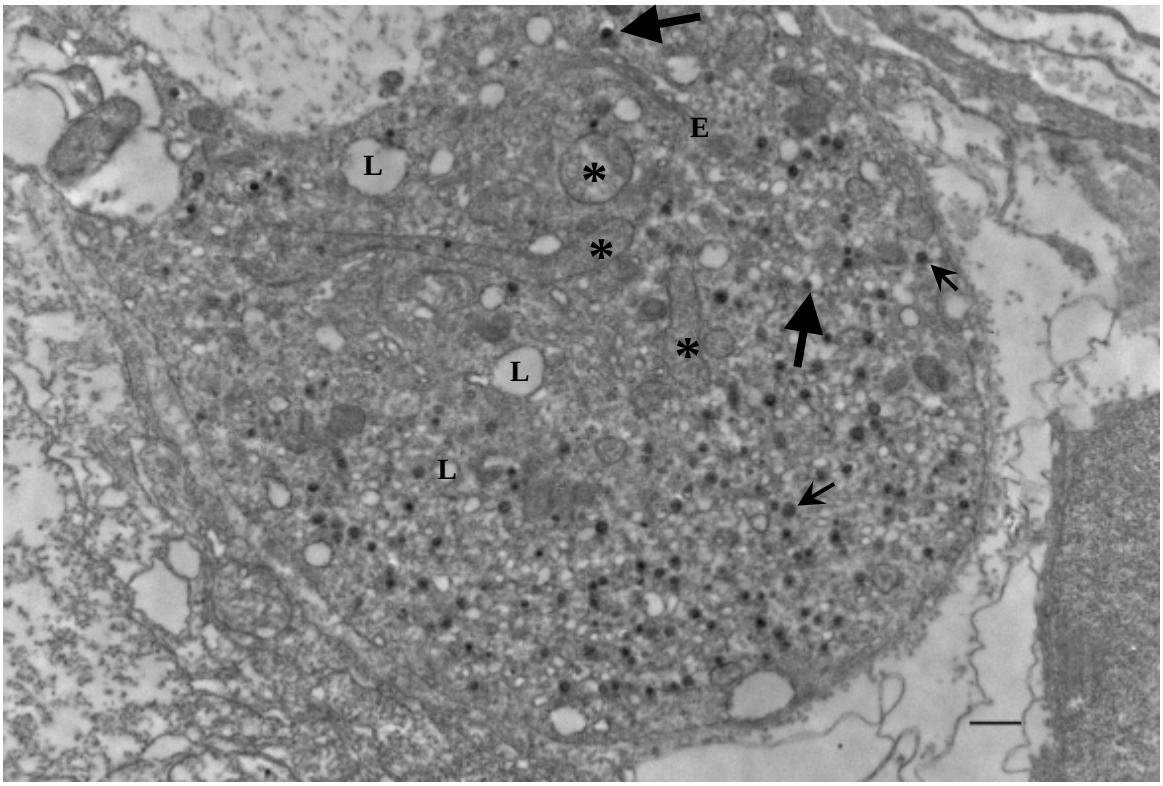


(e) 40,000X. Bar = 100nm.

Figure 3.16: TEM views of pheochromoblast related to the neural tube. (a) 6.3X, 1.5µm thick section of a 14 dph sturgeon pro-larva raised at 12°C showing the location of chromaffin cell related to the neural tube (NT). AC, alimentary canal; AK, anterior kidney; S, skin (insert). The section was stained with toluidine blue. (b) Elongated chromaffin cell (arrow head). (c) Chromaffin cell showing dispersed vesicles (arrows) along with mitochondria (*), lipid vacuoles (L), nucleoplasm (Np), nuclear membrane (Nm) and plasma memberane (Pm). (d) Cephalic end of chromaffin cell. (e) Higher magnified view of chromaffin cell.



(b) 1000X. Bar = 2µm.



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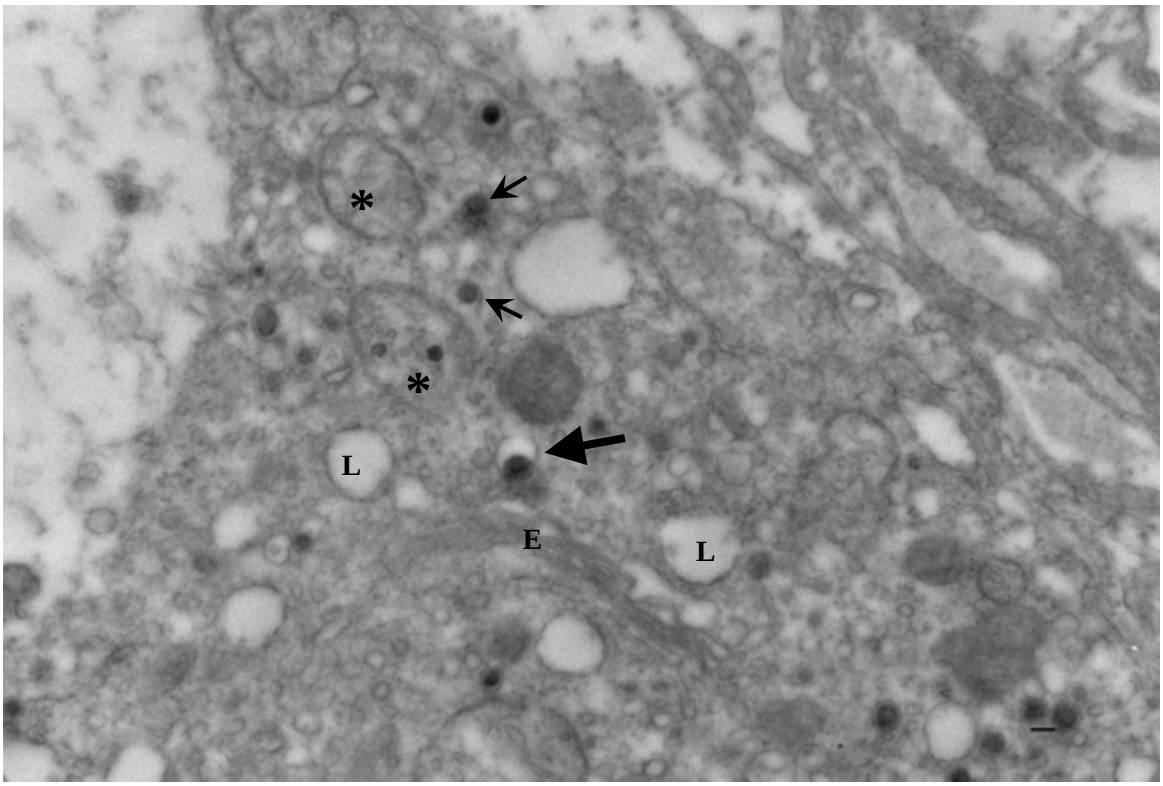
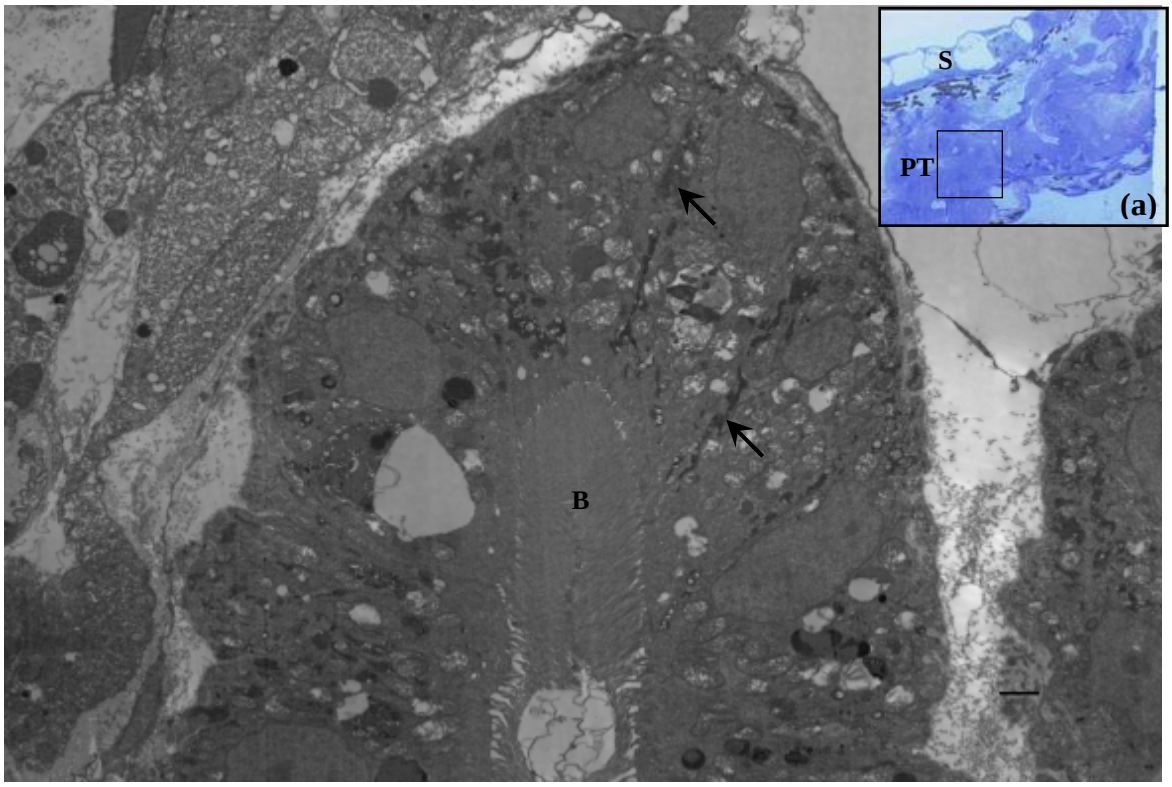
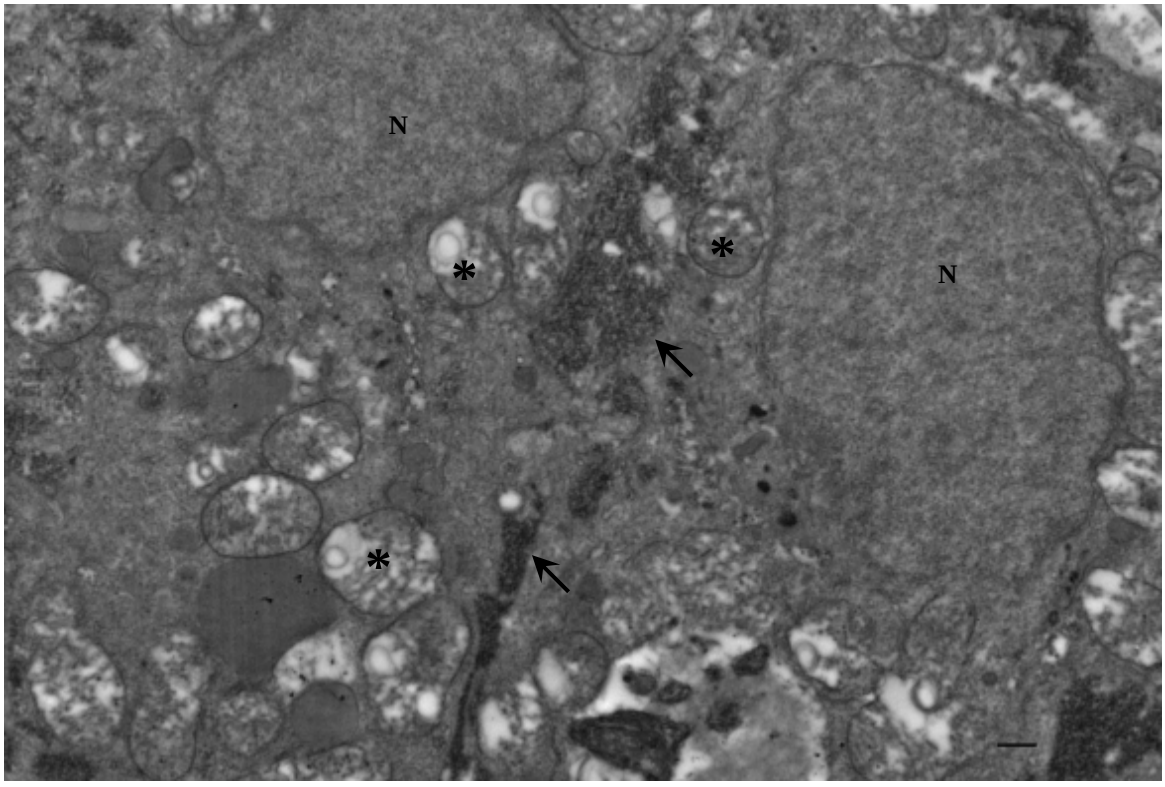


(d) 12,000X. Bar = 100nm.

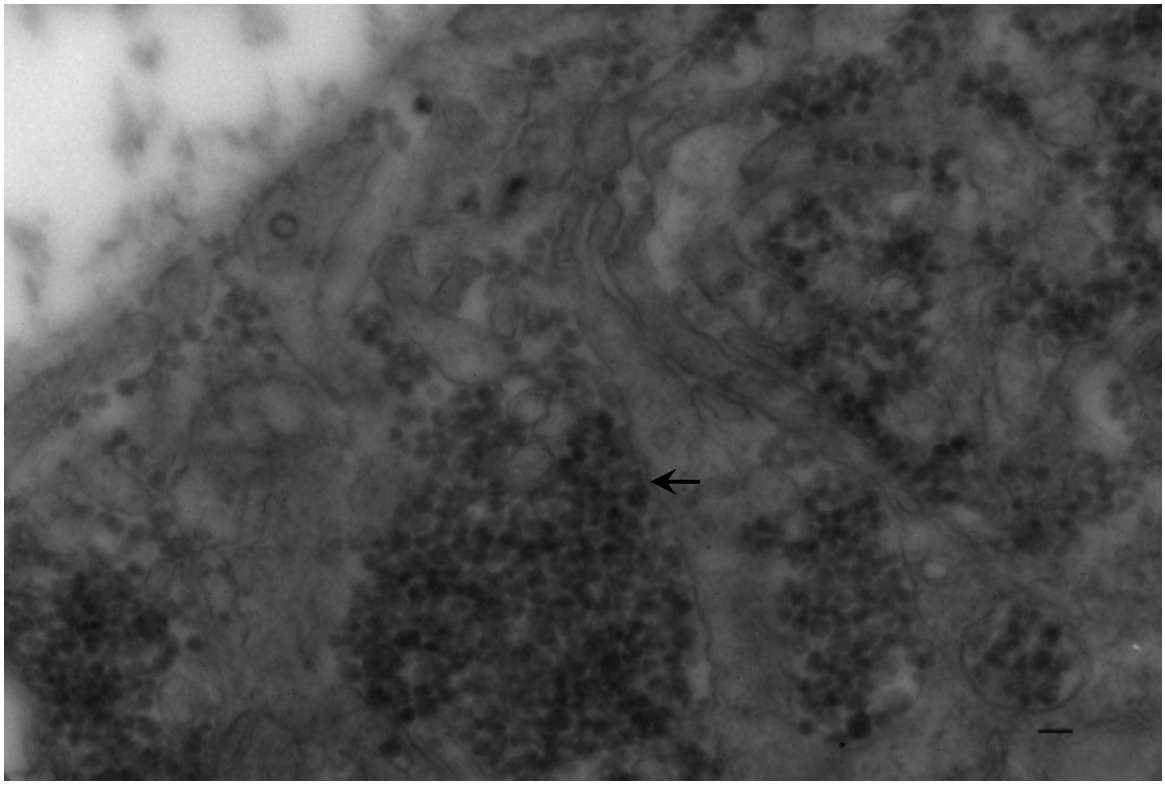
Figure 3.17: TEM views of pheochromoblast related to a blood vessel. (a) 6.3X, 1.5µm thick transverse views of a 14 dph sturgeon pro-larva raised at 12°C showing the location of the alimentary canal (AC), anterior kidney (AK) and neural tube (NT) (inset). The section was stained with toluidine blue. (b) Elongated chromaffin-like cell (arrow) related to the blood vessel (BV). (c) Chromaffin cytoplasm showing dispersed epinephrine (thin arrows) and norepinephrine (thick arrows) like vesicles along with mitochondira (*), endoplasmic reticulum (E) and lipid vacuoles (L). (d) Higher magnified view of chromaffin cell.



(b) 1000X. Bar = 2µm.

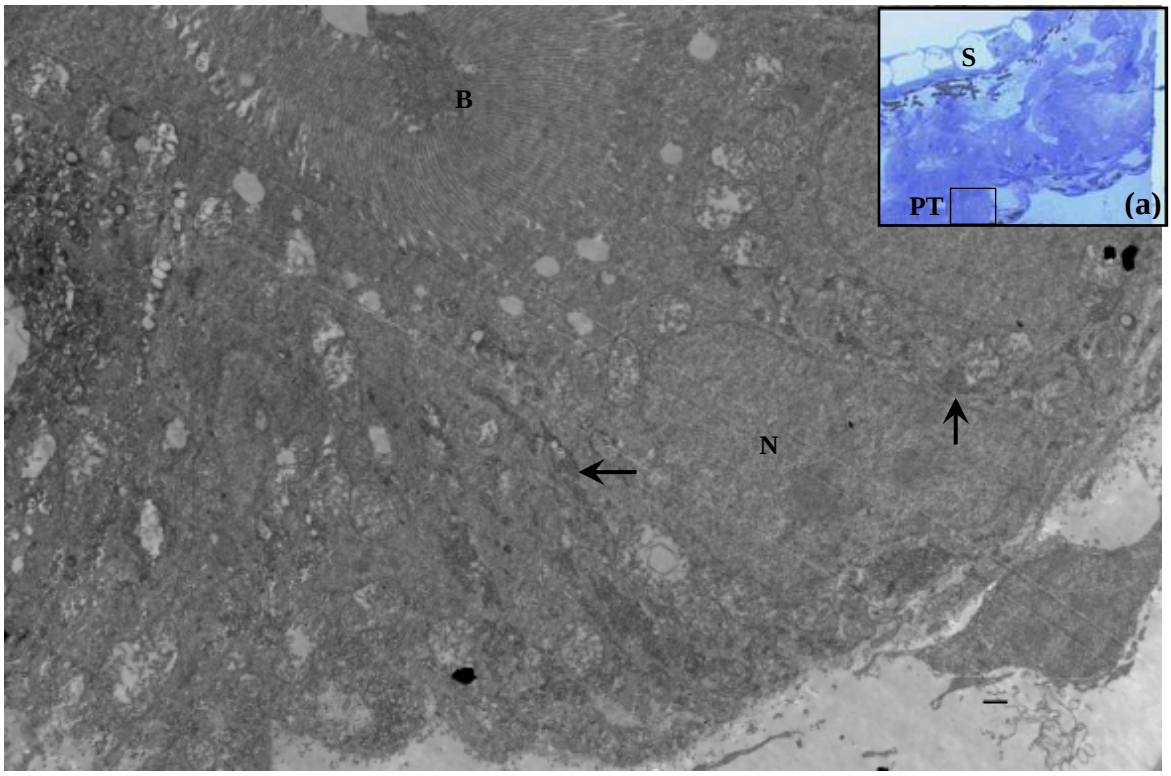


(c) 4000X. Bar = 500nm.

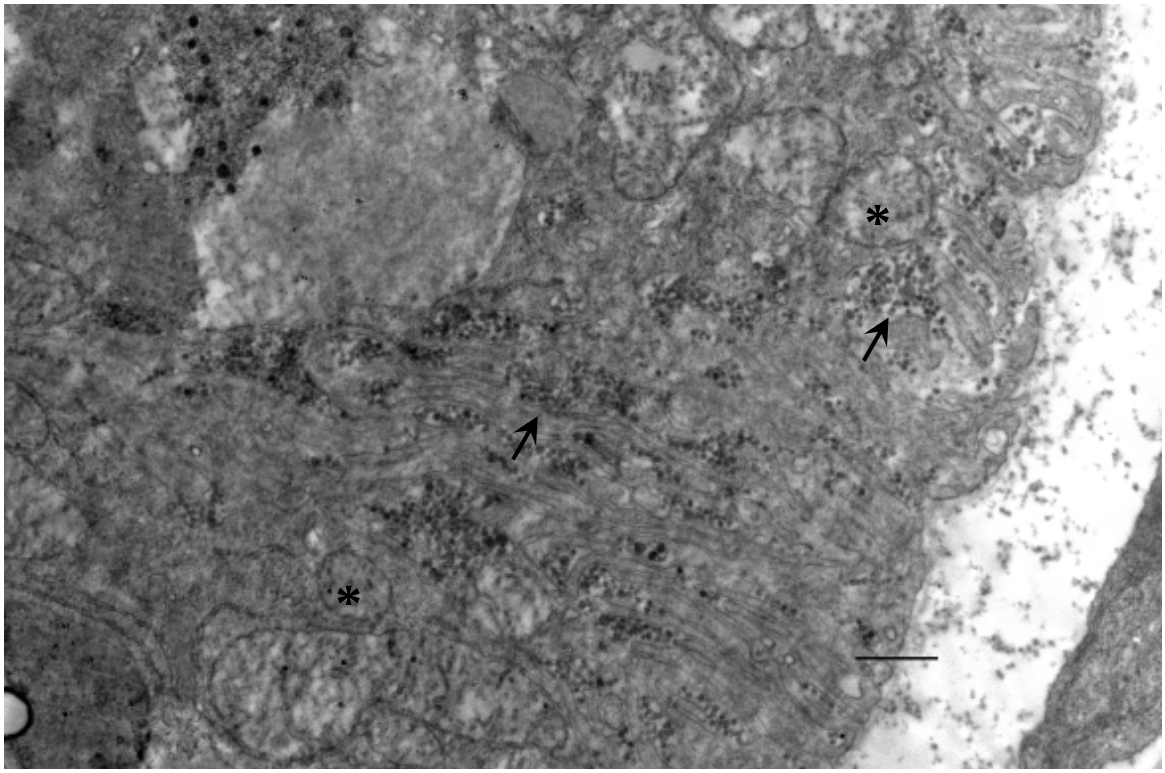


(d) 17,000X. Bar = 100nm.

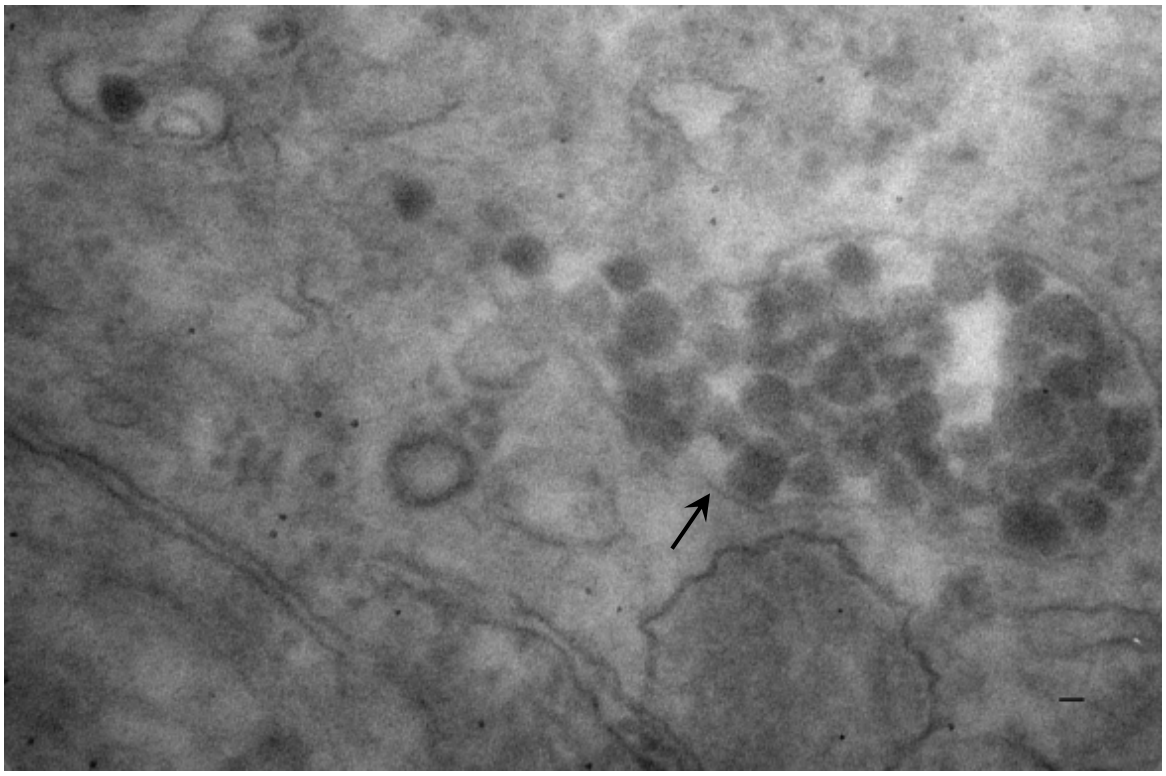
Figure 3.18: TEM views of chromaffin-like vesicles related to the proximal tubule. (a) 20X, 1.5µm thick views of a 14 dph sturgeon pro-larva raised at 12°C showing the location of the proximal tubule (PT) and skin (S) (inset). Section was stained with toluidine blue. (b) Proximal tubule of kidney recognized by the brush border (B) showing epinephrine like vesicles filled sacs (arrows) running from the basal to the apical surface of the cell. (c) Proximal tubule cytoplasm showing sacs filled with epinephrine like vesicles (arrows) along with mitochondria (*) and nucleus (N). (d) Ultra-structure showing invaginations of plasma membrane and forming sacs filled with epinephrine like vesicles.



(b) 2500X. Bar = 500nm.



(c) 8000X. Bar = 500nm.



(d) 60,000X. Bar = 20nm.

Figure 3.19: TEM views of chromaffin-like vesicles related to the proximal tubule. (a) 20X, 1.5µm thick views of a 14 dph sturgeon pro-larva raised at 12°C showing the location of the proximal tubule (PT) and skin (S) (inset). The section was stained with toluidine blue. (b) Proximal tubule of kidney recognized by the brush border (B) showing epinephrine like vesicles filled sacs (arrows) running through the basal to the apical surface of the cell. N, nucleus. (c) Proximal tubule cytoplasm showing sacs filled with epinephrine like vesicles (arrows) along with mitochondria (*). (d) Ultra-structure of membrane bounded sac filled with epinephrine like vesicles.

3.4 – ***Discussion***

As stated the chromaffin reaction involves of the interaction of potassium dichromate with the amide groups of catecholamines and imparts a yellow to brown color under light microscopy. With the exception of the head kidney control images from the goldfish, the present study demonstrated a dark brown to black color pigment at three major locations; under the skin, within the renal tissue and on the outer side of alimentary canal/yolk sac including the capsular area surrounding the kidney. Interestingly these pigments were also seen in tissues fixed in PF and modified Karnvosky's fixative. Despite an obvious reaction occurring in the staining technique used, given the observed and consistent locations of the dark brown to black stain it is unlikely that this tissue was chromaffin tissue. With respect to location, the pigment observed just under the skin is most likely representative of melanin pigment associated with melanocytes that one would expect in these locations (Leclercq *et al.*, 2010). This has also been observed in previous studies using similar staining techniques. In rainbow trout the appearance of dark brown to black pigments in renal tissue (Anderson and Loewen, 1975) were subsequently identified as melanin (Oguri, 1976). Finally the dark staining observed in the capsular area surrounding the kidney may be one of three possibilities all of which are known to impart a dark brown to black stain, hemosiderin, lipofuschins and melanin. While it is not possible in the present study to precisely determine which of these pigments may have generated the observed staining future studies employing Bunting's prussian's blue test, sudan black B stain or a bleach test by hydrogen peroxide would provide further information on the nature of these observed pigments (Oguri, 1976).

The chromaffin reaction was first introduced by Joesten (1864) and requires an optimal pH of 5 – 6 (Hillarp and Hokfelt, 1955). In more acidic environments EPI fails to react with dichromate unless the tissue has been previously fixed with glutaraldehyde (Coupland and Hopwood, 1966, Wood and Barrnett, 1964). Given that larvae in the present study were not fixed with glutaraldehyde prior to fixation with DF it may be that the solutions used were not at an appropriate pH for an optimal reaction to occur, however this is difficult to confirm. A second possibility for the lack of identification

using the chromaffin cells may be that the chromaffin-like cells identified from the electron microscopy images were not sufficiently abundant to provide the resolution required for locating them using the chromaffin reaction. Interestingly difficulties in identifying chromaffin tissue using the chromaffin reaction in this study have previously been reported (De Smet, 1970, see Gallo *et al.*, 2004). Therefore it may be that during the early stages of larval development chromaffin tissue is not prevalent in this species. For confirmation there are two avenues that one could follow. The first would be to utilize a highly specific technique to identify the chromaffin cells such as immunohistochemistry. Secondly the chromaffin reaction could be used on more mature juvenile lake strugeons where one would anticipate the abundance of chromaffin cells to be higher for confirmation of whether the chromaffin reaction provides sufficient resolution to identify chromaffin cells in lake sturgeon.

Various enzymes of the Blaschko pathway in a number of organs have been recognised in teleost embryo's, larvae (Ekstrom *et al.*, 1992, Gallo and Civinini, 2001) and adults (Dumbarton *et al.*, 2010, Gallo *et al.*, 2001). The use of antibodies raised against these enzymes has been shown to be highly specific in nature and thus extremely useful for the identification of chromaffin tissue in larval fish. Milano *et al.* (1997) studied the chromaffin system in Salmonids, Perciformes and Cyrinodontiformes at various stages of development. Using immunohistochemical techniques these researchers located cells positive for DBH and PNMT activity much earlier than was possible using the chromaffin reaction described in the present study and a similar study has utilized such antibodies in the beluga sturgeon (Gallo, *et. al*., 2004). In Salmonids localization of enzymes was determined close to hatch, whereas the chromaffin reaction was not effective for another 14 days. In comparison, Perciformes showed a delay in the appearance of the enzymes associated with NE and EPI synthesis and were not evident by immunohistochemical means until 20 dph in cells related to the PCV and sympathetic ganglion, however, the chromaffin reaction was again not sufficiently sensitive for a further 10 days. At 35 dph cells of sympathetic ganglion were seen completely diffuse with the chromaffin cells related to PCV indicating the final development of the adrenergic system. Similarly in Cyprinodontiformes enzymes did not appear until the complete absorption of the yolk sac. Gallo and Civinini (2005) studied the development

of the chromaffin system in rainbow trout by immunohistochemical and ultra-structural means. They found a similar enzymatic activity at 27 dpf near the time of hatching consistent with the previous study. Ultra-structurally chromaffin cells at this stage contained a single type of granule with a large nucleus and had minimal innervations. Two different types of chromafin cells were identifiable only after 5 dph, and there was a well developed cholinergic innervation at 10 dph. Chromaffin cells acquired the features of adult fish at around 60 dph.

In light of these studies the light and electron microscopy results of the present study indicate that lake sturgeon chromaffin development at 14 dph may be considered equivalent to 1 to 2 dph in the developing rainbow trout. Chromaffin cells associated with the neural tube are spindle shaped with one end in the images examined being more pointed than the other. This can represent the sympathoadrenal cell lineage where cells originating from the neural crest cells migrate to the targeted adrenal gland in mammals (Unsicker *et al.*, 1997) and head kidney in teleost fish (Gallo and Civinini, 2005). The presumed polymorphic shape of the chromaffin cells in the present study along with the variably dense vesicles and lack of innervation suggest these cells may be immature. Furthermore, these chromaffin-like cells associated with the neural tube had a very large nucleus that typically occupied much of the cytoplasm suggesting that they may be equivalent, from a development standpoint to pheochromoblastic cells in mammals (Kent and Parker, 1993, Unsicker *et al.*, 1985).

The finding of chromaffin-like vesicles associated with the PT of the kidney was a surprise. Although they were not found in the rest of the kidney tubule the vesicles were smaller in size than the other chromaffin-like cell type. These findings along with variable electron densities may indicate a mixture of various monoamines in the contents of the vesicles. Immunohistochemical alongside fluorescence histochemistry and biochemical estimation may help in determining the vesicular contents of this cell type.

It is well recognized that a change in temperature is perhaps the most pervasive environmental influence on metabolic rate of all vertebrate organisms (Gillooly *et al.*, 2001) and this has been demonstrated in regard to hatching rate in a number of teleost

species (Heggberget, 1988, Pepin *et al.*, 1997). The present study clearly demonstrates a similar effect on the development of pro-larval and larval lake sturgeon where the 15°C treatment group hatched 9 days prior to the 9°C treatment group and began feeding 26 days prior to the 9°C treatment group. In the wild, lake sturgeon embryos develop over a gravel substrate (Bruch and Binkowski, 2002) and burrow into the interstitial spaces following hatch, where they remain until emergence and the onset of exogenous feeding (Auer and Baker, 2002, Smith and King, 2005). In addition to the obvious hatch and feeding developmental milestones the development of renal structures also showed considerable differences in relation to the temperature treatments, with the 15°C treatment group being considerably faster than the 9°C treatment group, both in regard to the proliferation of the kidney but also in the appearance of distinct anatomical differentiation of the renal tubule.

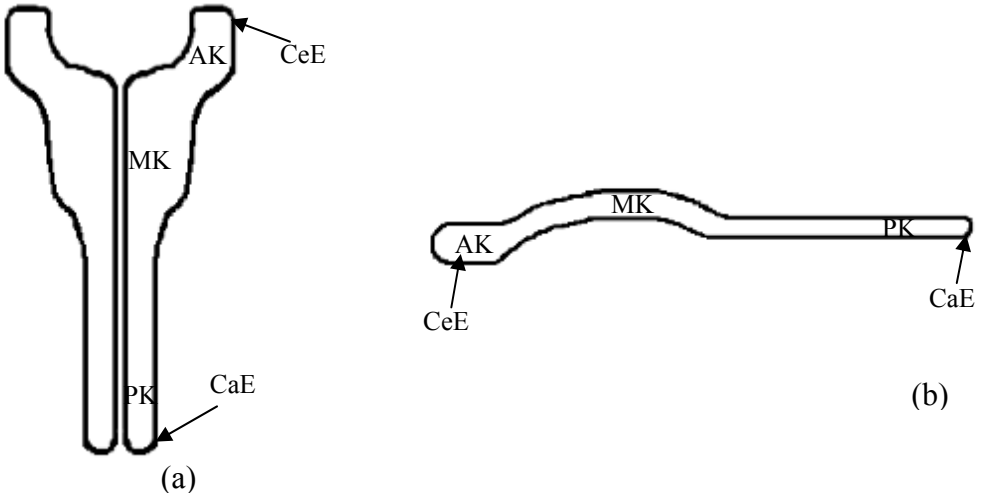


Figure 3.20: Schematic representation of coronal (a) and lateral (b) views of Acipenser fulvescens kidneys at larval stage. AK, anterior kidney; MK, middle kidney; PK, posterior kidney; CeE, cephalic end; CaE, caudal end.

Figure 3.20 is a schematic representation of the kidney in a fully grown lake sturgeon larva and figure 3.21 shows the relative growth of the kidney at 11 dph in pro-larvae raised at the different temperatures used in the present study. In a typical pro-larva all the kidney tubules and CDs are arranged at specific locations in the whole kidney. The anterior kidney consists of the PnT and APT towards the dorso medial axis, the PPT towards the dorso lateral axis, the DT towards the ventro medial

axis and the CD towards the ventro lateral positions (figure 3.15a-e). The middle kidney is relatively flat consisting of the PnT, APT, PPT, DT and CD from medial to lateral positions (figure 3.15f-p). The posterior kidney is relatively cylindrical and seems to contain only the PnT and supporting tissue (figure 3.7e-f, 3.13e-f). Although a blood vessel related to the kidney was identified in a 27 dph larva raised at 12°C (figure 3.10d) it was not possible to further explore this due to the poor fixation of the DF reagent. In adult sturgeon the posterior kidneys are united (Gambaryan, 1988, Mok, 1981, Ogawa, 1961, 1962), however, in the present study the posterior regions of the kidneys were separated at the pro-larval and larval stages.

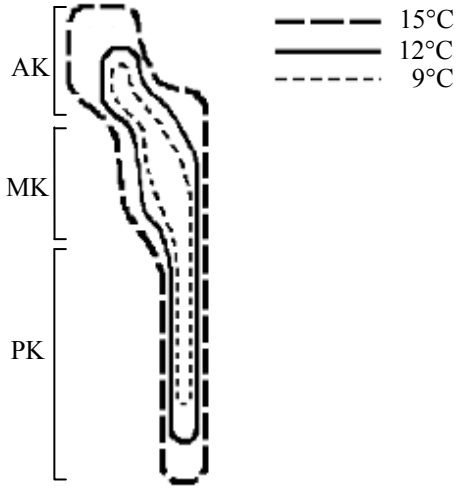


Figure 3.21: Relative size and shape of kidneys of a typical 11dph Acipenser fulvescens pro-larvae raised at 15°, 12°C and 9°C.

The PnT represents the initial stages of kidney development and is generally present in embryological and pro-larval stages and differentiates to form the various tubular structures as the fish develops. The timing of this development appears to be entirely dependent on the various fish species (Varsamos *et al.*, 2005). Despite the lake sturgeon being a freshwater species glomeruli were typically absent at the pro-larval stage. Interestingly, appearance of glomeruli during development seems to be variable in teleost fishes. In chum salmon, *Oncorhynchus keta*, guppy, *Lebistes reticulatus*, Atlantic herring, *Clupea harengus* and zebrafish, *Danio rerio*, the glomeruli are fully developed glomeruli at the time of hatching (Agarwal and John, 1988, Drummond *et al.*, 1998,

Takahashi *et al.*, 1978, Tytler *et al.*, 1996), however, in turbot, *Scophthalmus maximus* and European seabass glomeruli are not evident until 5 and 2 dph respectively (Nebel *et al.*, 2005, Tytler *et al.*, 1996). Using light microscopy, Abadi *et al*. (2011) illustrated the renal structure of the Persian sturgeon, *Acipenser persicus* during development, while the exact timing of the appearance of glomeruli was not reported the glomeruli were arranged on the ventral line of the kidney at the larval stage. In addition to the glomeruli, most kidneys in fresh water fishes have a neck segment and intermediate tubule. The neck segment is typically a short potion of the kidney tubule present between the glomerulus and PT and has been identified in many fresh water teleost species (Hickman and Trump, 1969), in addition to paddlefish, (Krayushkina *et al.*, 2000) and the Persian and Adriatic sturgeon (Abadi *et al.*, 2011, Khodabandeh *et al.*, 2009, Ojeda *et al.*, 2003). The intermediate tubule is present between the PT and DT and is present in some teleosts (Hickman and Trump, 1969) and sturgeons (Gambaryan, 1988). These two structures were not found in the present study even at the larval stage and studies on juvenile fish are recommended to determine if the nephron of lake sturgeon possess either the neck segment or intermediate tubule.

In most of the pro-larvae and larvae, the PnT showed changes in the morphological features even in the same kidney. Normally the PnT is differentiated from the rest of tubule by having brush border, smaller diameter of the lumen, low columnar epithelium and fewer convolutions (figure 3.15m). However, in some of the sections brush border was lost (figure 3.15h) while in other sections there was vacuolization observed in the epithelial cells lining the PnT with formation of debris in the lumen along with loss of the brush border (figure 3.15d). Such features have also been observed in tubules with ‘acute kidney injury’ (AKI). Hentschel *et al.* (2005) studied AKI in larval zebrafish and found similar morphological features in the PnT following administration of gentamicin as a nephrotoxic drug. Along with the 15°C treatment group these features were also observed in larvae raised at 12° (figure 3.7 c to f) and 9°C (not shown in figures). These findings suggest that the cause of AKI was not related to a change in environmental factors and therefore maybe the result of some unknown environmental factors.

PT in present study were differentiated into the APT and PPT with the reduced lumen of the APT being the only positive mode of differentiating between the two regions of the PT. However, in the developing larvae of the Persian sturgeon in addition to a reduced lumen the columnar cells of the APT were taller with a distinct brush border, whereas the PPT cells were more cuboidal and had microvilli on the luminal membrane (Abadi *et al.*, 2011). In contrast, studies performed on *P. spathula* and beluga sturgeon showed only one PT segment (Krayushkina *et al.*, 1996a, Krayushkina *et al.*, 2000). The DT and CD were less convoluted and did not contain a brush border in the lake sturgeon larvae, were located more laterally and ventrally and represented the end of the tubular system. Krayushkina *et al.* (1996) did not find significant differences in between DT and CD of beluga sturgeon and thus they named the CD as the terminal region of the DT. Further differences in these two regions of the kidney tubule of lake sturgeon could be evaluated by ultra-structural and immunofluorescence techniques.

Chapter 4: Conclusion

In conclusion, the present study shows some significant changes in both NE and EPI post-stress in both the solitary and grouped treatments. However, comparison between treatments was somewhat in-conclusive and would be improved with an increased n value for each treatment. In a previous study, Allen *et al.*, (2009) demonstrated an effect of sociality on the cortisol stress response where isolation increased the duration of the cortisol stress response. However, it is not understood if the shortened stress response in grouped fish was due to the presence of con-specifics in the experimental tank or if the presence of allo-specifics at the same density would result in a similar shortened stress response as seen in the grouped treatment. Furthermore, measurement of catecholamine concentrations in lake sturgeon juveniles in the presence of conspecifics (this study) or allo-specifics would provide further support for a physiological benefit to living in large aggregations. In the Winnipeg River juvenile lake sturgeon are known to aggregate at very high densities in deep main channel habitat (Barth *et al.*, 2011) suggesting an ecological benefit in social grouping. From an ecological perspective it is likely that trade-offs exists between high density, resource availability, predation and growth. However, from a physiological perspective the data in this thesis, supported by previously published work (Allen *et al.*, 2009) suggests that social grouping may well confer benefits in both the acute and chronic stress response. Further experiments to explore the relationship between social interaction and the stress response could include examination of the stress response in juvenile lake sturgeon in the presence of a more pelagic species, such as rainbow trout or in the presence of a species that may share similar habitat such as channel catfish.

In regard to the second experimental chapter which is speculative at this stage, presumptive pheochromoblasts identified at 14 dph appeared plemorphic in shape and contained mixed vesicles that were located lateral to the neural tube and near the blood vessels related to the kidney perhaps representing the stage of movement along the sympathoadrenal cell lineage. The chromaffin reaction was not positive representing the immaturity of the adrenal system or lack of sensitivity of this method in this life stage. Further studies via immunohistochemical and ultra-structural means at various age

groups would provide a more definitive answer in regard to the timing of appearance of both potentially immature and mature chromaffin-like cells. Chromaffin-like vesicles related to the PT were entirely of unknown origin. Although the ultra-structure was not similar to the vesicles observed in pheochromablast further studies via immunohistochemical, fluorescence histochemistry and biochemical estimation would allow for appropriate identification of this cell type.

Temperature did not have any effect on the pattern of kidney development in the present study, however, the timing of appearance of more developed tubular segments was affected by temperature, with the 9°C treatment group being significantly slower in development than the 12 and 15°C treatments. Additional ultra-structural studies would better describe the differences between tubule structures at a sub-cellular level and provide further information in regard to the function of the described segments.

Appendix: List of Abbreviations

Acute kidney injury.....AKI
Adrenocorticotropic hormone.....ACTH
Anterior proximal tubule.....APT
Collecting duct.....CD
Corticosteroid receptors.....CR
Corticotropin releasing hormone.....CRH
Day post fertilization.....dpf
Day post hatch.....dph
Dichromate fixative.....DF
Dihydroxyphenylalanine.....DOPA
Distal tubule.....DT
Dopamine.....Dp
Dopamine β-hydroxylase.....DBH
Epinephrine.....EPI
Ethyl alcohol.....EA
Glucoocorticoid recreptors.....GR
Hematoxylin and eosin.....H&E
Hypothalamic-pituitary-interrenal.....HPI
Mineralocorticoid receptors.....MR
Norepinephrine.....NE
Paraformaldehyde.....PF
Phenylethanolamine N-methyltransferase.....PNMT
Posterior cardinal vein.....PCV
Posterior proximal tubule.....PPT
Pronephric tubule.....PnT
Proximal tubule.....PT
Standard error.....S.E
Transmission electron microscope.....TEM
Tricaine methanesulphonate.....MS222
Tyrosine hydroxylase.....TH

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